Diazepam binding inhibitor overexpression in mice causes hydrocephalus, decreases plasticity in excitatory synapses and impairs hippocampus-dependent learning

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Diazepam binding inhibitor (DBI) and its processing products are endogenous modulators of GABA_A and linked to various brain disorders ranging from anxiety and drug dependence to epilepsy. To investigate the physiological role of endogenously expressed DBI in the brain we created a transgenic mouse line overexpressing *DBI* gene. Transgenic mice had a 37× increased protein expression and immunohistochemistry showed excessive glial expression in the infragranular region of the dentate gyrus. Transgenic animals had significantly larger lateral ventricles and decreased plasticity of excitatory synapses without affecting either inhibitory or excitatory synaptic transmission. In behavioral tests transgenic animals had no differences in motor and exploratory activity, yet impaired hippocampus-dependent learning and memory. Overexpression did not cause anxiety or proconflict behavior, nor influenced kainic acid or pentylenetetrazole induced seizure activity. Our transgenic mouse line demonstrates that endogenously overexpressed DBI impairs hippocampus-dependent learning without anxiety or proconflict behavior.
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Introduction

Diazepam binding inhibitor (DBI) is a 10 kDa protein first purified from rat brain (Guidotti et al., 1983). It was isolated by its ability to displace diazepam from type A γ -aminobutyrate receptors (GABA_A receptors) and mitochondrial benzodiazepine receptors. The same protein has been shown to bind selectively to medium and long chain acyl-CoAs with high affinity, hence named as acyl-CoA binding protein (Faergemann and Knudsen, 1997).

In brain, DBI and its two processing products octadecaneuropeptide (ODN; DBI_{33–50}) and triakontatetrapeptide (TTN; DBI_{17–50}) are expressed unevenly, with the highest levels found in hypothalamus, amygdala and cerebellum (Alho et al., 1985; Ferrarese et al., 1989). The two processing products differ in both biological activity and physicochemical properties. TTN binds preferentially to the benzodiazepine receptors and to the allosteric site of the GABA_A receptor, whereas ODN binds to the allosteric modulatory center of GABA_A receptors at which benzodiazepines (BZDs) bind (Berkovich et al., 1990). The biological action of DBI and its processing products is mediated by their ability to modify GABA_A receptor function, either directly through their ability to replace BZDs or through neurosteroids that act on recognition sites located in the transmembrane region (Costa and Guidotti, 1991). DBI acts as a negative allosteric modulator of GABA_A receptors and is acting as a proconflict agent (Bormann, 1988). Both ODN and TTN have been found to be more potent than DBI in inducing proconflict behavior (Ferrero et al., 1986; Slobodyansky et al., 1989). In humans DBI-like immunoreactivity (DBI-LI) has been

Abbreviations: TG, transgenic (tg); SG, syngenic (sg); DBI, diazepam binding inhibitor; ODN, octadecaneuropeptide; TTN, triakontatetrapeptide; PTZ, pentylenetetrazole; KA, kainate; LTP, long-term potentiation.

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measured from cerebrospinal fluid of patients suffering from disorders linked to abnormal GABAergic transmission. DBI levels were found to be increased in patients suffering severe anxiety, however, they were not changed in disorders like multi-infarct dementia or dementia with Parkinson's disease (Barbaccia et al., 1986; Ferrero et al., 1988). In Alzheimer disease, higher DBI levels have been reported in the cerebrospinal fluid by Ferrarese et al. (1990), while Ferrero et al. (1988) and Barbaccia et al. (1986) found no difference. Recently, Tokay et al. (2005) demonstrated that beta-amyloid peptides stimulate ODN-LI from cultured rat astrocytes.

In addition to its involvement in anxiety and aggressive behavior, DBI and its processing products have been postulated to play a role in epilepsy. Injections of DBI_{42–50} and DBI_{43–50} into the dorsal hippocampus of rats induced the typical patterns of limbic seizures (Vezzani et al., 1991). Furthermore, significantly higher DBI plasma concentrations have been found in epileptic patients (Ferrarese et al., 1998). Strikingly, plasma DBI levels were much higher in generalized epilepsy in adults and in drug-resistant patients.

Since previous studies have revealed varying and conflicting results, we investigated the function of DBI in a physiological setting using transgene technology. We isolated the gene and generated a transgenic mouse line overexpressing DBI. In this model we investigated the phenotypic changes and analyzed the seizure threshold using kainate acid (KA) and pentylenetetrazole (PTZ). Furthermore, we investigated by long-term potentiation (LTP) whether transgenic mice show alterations in excitatory synaptic transmission or potentiation in the CA1 field of the hippocampus.

Results

Mouse DBI gene

The isolated mouse *DBI* gene (11.1 kb) consisted of 1.8 kb promoter, 4 exon regions including the recently found alternative exon 1 (Nitz et al., 2005) and 1.9 kb 3' flanking region. The promoter region contained various potential binding sites for transcription factors including peroxisome proliferator activated receptors (PPARs) that have been previously reported to take part in regulation of DBI expression (Helledie et al., 2002). The exon/intron structure was similar to that of the rat and human genes. Comparison of the exon sequences with mouse DBI cDNA revealed no base differences on the protein coding region. The nucleotide sequence of the isolated mouse

DBI gene has been submitted to GenBank database under accession number AF22022.

Generation of transgenic mice

Microinjections created two mouse lines carrying the DBI transgene. The line designated UKU302F was chosen for further studies on basis of higher expression levels. Transgenic (tg) mice were viable and fertile. Quantitative real-time PCR from genomic DNA of the tg mice confirmed that there is a 30 times increase in *DBI* gene copy number (Fig. 1A). DBI mRNA levels were 6.2 times higher in the tg animals than in the sg littermates (Fig. 1B). Western blots from brain confirmed that the tg animals had 37 times more DBI protein than the sg littermates (Fig. 1C). Tg animals had elevated mRNA and protein levels also in other tissues studied (data not shown).

Histology results of cellular localization of DBI

Staining of mouse brain with DBI antibody showed excessive expression in the infragranular region of the dentate gyrus in tg animals (Fig. 2). Double staining with neuron-specific NeuN and glial cell marker GFAP demonstrated that both in the infragranular region as well as in the CA3 field of the hippocampus DBI was mainly expressed in glial cells (Fig. 3).

Morphological analysis of the ventricles

Mean ventricle areas were calculated for each mouse and compared between tg and sg animals by planimetric analysis. Adult tg mice had significantly larger mean ventricle area than sg controls (data not shown). In addition, the difference in ventricle size was confirmed by MRI study showing that the adult tg animals had a 5 times bigger mean ventricle area than their sg littermates (Fig. 4). MRI studies with new born mice revealed that the differences were already evident at birth (data not shown) and evaluation of a rat line overexpressing the same transgene confirmed that the hydrocephalus is not caused by an insertional mutagenesis (Herzig et al., unpublished data).

Behavioral testing

Evaluation of the behavioral profile showed that there was no major difference between the groups in motor and exploratory activity as assessed by open field, elevated plus maze, light–dark and Y-maze tests. Furthermore, the pain sensitivity (hot plate) and

Fig. 1. Brain DBI overexpression at gene (A), transcriptional (B) and protein (C) level.

Fig. 2. DBI in the hippocampus. Sections from tg (A) and sg (B) (10 $\times$, scale bar 200 μ m) mice stained with DBI antibody. Arrows in tg mouse point heavily stained cells in the infragranular region of the dentate gyrus. H=hilus, Mol=molecular layer, Gr=granule cell layer.

coordination (rotarod, beam balancing) were not affected by the overexpression of the DBI transgene (data not shown). Yet, in the fear conditioning experiments tg males displayed significantly reduced freezing in conditioning context (hippocampus-dependent form of memory) as compared with sg males (Fig. 5). However, there was no difference between females (effect of genotype $F(1,31)=3.6$, $p=0.067$; interaction of genotype and sex $F(1,31)=6.8$, $p=0.014$). The freezing in response to the conditioned stimulus (tone; amygdala-dependent) was not different between the sg and tg mice.

All groups learned to find the hidden escape platform in the water maze experiment (Fig. 6A). However, in the first transfer test (after 4 learning blocks) the sg mice displayed a clear preference to the trained quadrant whereas the tg mice did not show spatial selectivity (Fig. 6B). After prolonged training (2 additional blocks of trials) tg mice learned to search in the trained quadrant as well (Fig. 6C). Both groups were able to learn to find the platform in the new (opposite) location and showed preference to the new zone in the third transfer test (Fig. 6D). All mice rapidly learned to swim to the platform made visible by local cue confirming sufficient motor and motivational abilities to perform the swimming navigation task.

Evaluation of synaptic transmission and induction of long-term potentiation (LTP)

Field EPSP waveforms evoked by Schaffer-collateral stimulation were measured in CA1 during the baseline recording period. Comparable stimulation currents elicited equivalent amplitude responses in tg and sg slices and no differences in responses were observed between groups in any parameter tested (amplitude, slope, half-width; n.s.) (Table 1).

Theta burst stimulation led to a transient posttetanic potentiation up to 3 min in both sg and tg mice. In contrast, it leads to a long-term potentiation of synaptic strength only in sg but not in tg mice. In sg mice, Schaffer-collateral synapses exhibited approximately 100% increase in EPSP amplitude measured 10–45 min after high-frequency stimulation whereas the potentiation was approximately 20% in DBI tg mice during this time period (Fig. 7). There was no difference between sexes (for gender effect $F=0.06$, n.s.). Thus, there were significant differences in the long-term potentiation of EPSP amplitude between female tg and sg mice (for group effect $F=5.20$, $p<0.05$) and between male tg and sg mice (for group effect $F=6.13$, $p<0.05$). In all mice there were significant differences in the long-term potentiation of EPSP

amplitude between tg and sg mice (for group effect $F=11.21$, $p<0.01$) (Fig. 7).

Seizure induction with kainic acid (KA) and pentylenetetrazole (PTZ)

All mice treated with different doses of KA (Table 2) except one sg female (30 mg/kg) developed seizures during the 4-h observation period. The highest mortality (38.5%) in tg mice was at 28 mg/kg. In sg mice, the highest mortality (58.3%) was at 30 mg/kg. The time period from injection to death was the longest at the lowest dose 26 mg/kg used in both tg and sg animals. The time became shorter as the dose was raised in tg mice which was not observed in sg animals, yet the difference was not significant. Furthermore, there were no significant differences between sg and tg mice in the total numbers of seizures nor in the latency to first seizure during the 4-h observation period (Table 3). There was no difference in the amount of degenerating neurons in granule, hilus, CA3a, CA3b, CA3a and CA1 areas (Table 4).

All mice injected with 60 mg/kg PTZ developed seizures. Sg animals had higher mortality (37%) than tg mice (0%) but there were no statistical differences in either number or time period to the first seizure.

Discussion

The role of Diazepam binding inhibitor in the CNS is still unclear. Therefore, we created a DBI overexpressing mouse line to investigate its functions in a physiological setting. We utilized the mouse endogenous gene and promoter to mimic the physiological expression pattern as close as possible in comparison to wild-type animals. Characterization of this mouse line at the mRNA and protein level revealed that the transgene is overexpressed in brain in addition to other tissues like liver, muscle and fat. We found a high DBI-like immunoreactivity in the hippocampal granular cell layer. Utilizing double staining methods with neuron-specific nuclear protein NeuN and specific glial cell marker GFAP in the hippocampus we found that DBI was expressed in glial cells. Thus, our findings are compatible with previous studies by Costa and Guidotti (1991) and Yanase et al. (2002).

Transgenic mice were viable and fertile yet revealed a clear difference in regard to their ventricle size compared to their syngenic littermates. The difference was already evident at birth. Histological analysis and MRI studies revealed a significant enlargement of lateral ventricles in tg animals. A rat line over-

Fig. 3. Colocalization of DBI in the hippocampus. Colocalization of DBI (Cy2; green) and NeuN (Cy5; red) of *tg animals* in mouse granular cell layer (A) and the CA3 area of hippocampus (B). Colocalization of DBI and GFAP (Cy5; red) in sg (C) and tg (D) mouse granular cell layer. Colocalization, demonstrated by yellow, shows that DBI is mainly expressed glial cell. 60×.

expressing the same transgene was found to have same phenotype thus confirming that the hydrocephalus is caused by high levels of DBI not insertional mutagenesis (Herzig et al., unpublished data). Previous studies have shown elevated CSF levels of DBI in patients with dementia and normal pressure hydrocephalus (Ferrero et al., 1988). A possible explanation for the development of hydrocephalus in DBI *tg* mice might be perivascular astrocytosis which has been described to cause hydrocephalus in TGF- β 1 overexpressing mice. TGF- β 1 has been suggested to play a central role in the pathogenesis of both congenital and acquired hydrocephalus (Crews et al., 2004). In addition, TGF- β 1 is acting partly through NMDA receptors (Gomes et al., 2005). NMDA

receptors have been found to influence DBI mRNA expression (Katsura et al., 2001). Furthermore, Alzheimer type II astrocytosis has been shown to be associated with an increase in the concentration of DBI (Giguere et al., 1990). The effect of DBI overexpression on spatial and emotional learning and memory was analyzed by LTP measurements and behavioral tests. Our LTP results indicate that synaptic transmission from CA1 was not affected as assessed by extracellular recordings in the CA1 area of the hippocampus of *tg* mice. The short-term synaptic plasticity was neither influenced since both *sg* and *tg* mice showed posttetanic potentiation. However, the presence of high levels of DBI interferes with the development

A)	SG	B)	TG
0.28 (12)		5.25 (97)
	1.37 (9)		5.21 (96)
	0.63 (6)		6.53 (15)
	1.64 (2)		4.57 (4)
	1.61 (1)		6.78 (3)
	Average		1.11
SD	0.61	SD	0.95

Fig. 4. Hydrocephalus in mouse. MRI pictures show enlarged ventricles in *tg* mice (B) when compared to *sg* littermates (A). MRI measurements of ventricle volumes (mm³), lateral and 3rd. 1.25×, scale bar 1000 μ m.

Fig. 5. Behavioral analysis I: percentage of freezing behavior in the context and cue tests (CS) of fear conditioning task.

of long-term synaptic plasticity in these synapses. The excitatory response in LTP is known to be dependent on the activation of NMDA receptors (Larson and Lynch, 1988). Since DBI affects the inhibitory function of GABA_A receptors (Ferrarese et al., 1987), one would assume that with higher DBI levels in tg animals the excitatory response would have been elevated or tg animals would have had a lower inhibitory response in LTP experiments. On the contrary, there were no differences in the inhibitory responses and all excitatory responses (EPSPs) were similar in basic synaptic transmission for a given stimulus between the sg and tg mice. However, using 'theta burst' pattern (TBS) a significant difference was detected. This decreased excitatory potentiation might be due to the regulation of NMDA receptor function. Activation of NMDA receptor has been found to enhance DBI mRNA expression in cerebral cortical neurons (Katsura et al., 2001). It

Table 1

Slope, amplitude and half width of EPSP (50% of maximum)

	sg (n=18)	tg (n=17)	F	P value
Slope (V/s)	-0.40±0.13	-0.38±0.14	0.081	0.63
Amplitude (mV)	-0.20±0.12	-0.19±0.10	0.077	0.84
Half width (ms)	6.61±2.53	7.84±5.02	0.508	0.37

Results are expressed as mean±SD.

is therefore possible that in tg mice the NMDA receptors function is downregulated to prevent further DBI expression.

Behavioral tests were made to confirm if the decrease in the level of potentiation would have any effect on the memory or learning abilities of the tg animals. The basic behavior pattern of DBI tg mice was not different from their sg littermates, nor were tg animals more aggressive when handled. These results differ from previous findings demonstrating that acute icv. administration of DBI, ODN or TTN caused significant behavioral changes. Icv. injection of DBI has been found to reverse anticonflict action of diazepam (Guidotti et al., 1983), and ODN inhibited social and agonistic behavior and increased the incidence and intensity of offensive aggression in dominant resident mouse (Kavaliers and Hirst, 1986). DBI expression has also been found to be elevated in long-term administration of alcohol, nicotine and morphine in mice (Katsura et al., 1995, 1998a,b,c). In humans, the common clinical symptom among patients with dependence on these substances is anxiety. In our animal model DBI is continuously overexpressed, so that we cannot exclude the possibility that our tg mice are adapted to increased protein levels. On the other hand, our results demonstrate that overexpression of DBI significantly affected

Fig. 6. Behavioral analysis II: (A) the escape latency to the platform in the water maze; (B–D) time spent swimming in the target zone (TR) and symmetrically located alternative zones (left/right=L/R, opposite=OP) in the 1st, 2nd and 3rd transfer tests, respectively. ** $p < 0.01$ by one-way repeated measures ANOVA compared to the time in the trained location.

Fig. 7. LTP in DBI mice. The initial slope of excitatory postsynaptic potential (EPSPs) before and after theta burst stimulation (TBS) in slices prepared from DBI tg mice and sg mice. TBS was given at time 0. Each point is normalized to the average value during the baseline period (100%). (A) Female mice. DBI tg mice (●) ($n=10$) and sg mice (○) ($n=10$). (B) Male mice. DBI tg mice (●) ($n=7$) and sg mice (○) ($n=8$). (C) All mice. DBI tg mice (●) ($n=17$) and sg mice (○) ($n=18$). Note the LTP of EPSPs 10–45 min after TBS in sg mice but not in tg mice.

hippocampus-dependent learning and memory, especially on males. The differences between the genotypes in memory tests are specific and not confounded by the defects in other behavioral domains assessed (exploratory activity, anxiety-like behavior, motor and nociceptive functions). The reason for possible sex difference needs further evaluation.

Seizure threshold experiments were performed utilizing KA and PTZ to assess the effect of high DBI levels of on seizures activity. KA is an established model of human temporal lobe epilepsy because of behavioral and pathological similarities (Ben-Ari, 1985; Cendes et al., 1995; Nadler, 1981), acting as an agonist of non-NMDA receptors, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors and kainate receptors (Michaelis, 1998). Activation of kainate receptors has been described to downregulate GABAergic inhibition (Clarke et al., 1997; Rodriguez-Moreno et al., 1997). In addition, PTZ as a GABA_A receptor antagonist has been utilized as another well-established seizure model. Since intrahippocampal injection of DBI fragments caused seizures (Vezzani et al., 1991), we expected that DBI tg mice would respond differently in comparison to their sg littermates. In contrast to the reported literature and our expectations, there was no significant difference in number, severity or duration of KA induced seizures between tg and sg animals. Furthermore, there was no difference in amount of degenerating neurons after KA

injections. The same results were obtained from PTZ injections with the exception that sg had higher mortality (37%) than tg mice (0%) indicating that our tg mice had even some kind of protective mechanism against PTZ induced mortality. Similar reduction in mortality was observed when moderate amounts of ODN were injected icv. prior to PTZ induced seizures (Garcia de Mateos-Verchere et al., 1999). The reason for this discrepancy is unknown and will be needed to be addressed in further studies on mechanisms on the adaptation to high DBI levels.

In conclusion, our results clearly show that transgenic overexpression of DBI under its endogenous promoter does not cause anxiety or any proconflict behavior in tg mice. Tg mice are characterized by hydrocephalus and changes in LPT and hippocampus-dependent learning and memory. Our newly established transgenic mice line provides an excellent model to investigate in a physiological way the function of this interesting protein and the mechanisms behind the described changes and possible adaptations.

Experimental methods

Isolation of mouse DBI gene

Mouse DBI gene was isolated from mouse strain 129/SvJ genomic library (Stratagene, La Jolla, CA, USA) by PCR screening method (Israel, 1993) using to primer pairs. First pair amplified a 523-bp region of the first intron of mouse DBI gene (GenBank acc. L76367). For the other primer pair, rat DBI gene was compared to mouse EST sequence database in GenBank using BLAST. One EST containing sequence originating from intron three of mouse DBI gene was found and primers were designed to amplify a region from intron three to exon four of the DBI gene. The genomic library was plated into 96-well cell culture plate at density 1000 pfu/well. Samples were analyzed by PCR and secondary screening was performed. After tertiary screening, phages from positive wells were plated on LB-agar and individual plaques were picked up for PCR analysis. Positive clones were selected and subcloned into Bluescript KS (Stratagene, La Jolla, CA). One positive clone was sequenced on both strands by primer walking and transposon-mediated DNA sequencing according to manufacturer's instructions (Template Generation System, Finnzymes, Finland).

Generation of transgenic animals

The isolated gene was microinjected using standard techniques into the pronuclei of zygotes derived from Balb/c $\times$ DBA/2 mice. Adult transgenic (tg) and syngenic littermates (sg) were used in this study. The experiments were approved by Institutional Animal Care Committee of the University of Kuopio and by the Provincial Government. Procedures were

Table 2

Number and age of the animals and dosage used in the KA induced seizure study

Dose (mg/kg)	Weights (g)	Mouse group	Age		
			5 months	8 months	Total
26	24.2–36.2	tg	3	6	9
		sg	–	6	6
28	23.4–30.0	tg f	5	8	13
		sg f	4	6	10
	27.5–39.7	tg m	8	–	8
		sg m	8	–	8
30	19.0–33.5	tg	5	7	12
		sg	5	7	12

Table 3

Latencies and total duration of seizures (in minutes) after KA injection in female (f) and male (m) mice

Dose (mg/kg)	Mouse group	Latency to 1. seizure of score 1 or 2		Latency to 1. seizure of score 3 or 4		Total duration of seizures	
		N	Mean±SD	N	Mean±SD	N	Mean±SD
26	tg f	9	5.67±3.71	9	34.89±23.69	9	105.56±56.28
	sg f	6	5.50±1.38	4	22.71±15.96	3	104.33±33.38
	P value		0.723		0.153		0.926
28	tg f	13	9.31±4.25	12	22.17±12.17	10	113.30±51.11
	sg f	10	9.30±7.70	10	17.00±14.99	7	159.14±45.21
	P value		0.264		0.262		0.193
	tg m	8	7.13±1.55	7	21.43±10.03	5	128.60±60.43
	sg m	8	6.75±2.82	7	27.71±16.82	7	158.29±66.39
	P value		0.562		0.482		0.530
30	tg	11	7.08±3.84	12	21.50±22.09	8	147.00±39.42
	sg	10	5.10±1.97	10	15.20±12.31	4	144.75±39.42
	P value		0.230		0.429		0.497
All doses	tg f	33	7.61±4.14	33	25.03±22.36	27	120.70±51.10
	sg f	26	6.81±5.22	24	16.79±13.03	14	143.29±48.44
	P value		0.171		0.054		0.248

conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC.

Western blotting

Total protein was extracted by homogenization in extraction buffer (25 mM TRIS, 0.1 mM EDTA, 1 mM DTT and protease inhibitor cocktail (Sigma, St. Luis, MO)). Concentration was measured with BIO RAD's protein assay (Hercules, CA). Equal amounts of proteins were loaded on 15% SDS-polyacrylamide gel and after electrophoresis, the proteins were transferred to PVDF membrane. The blots were subsequently probed with DBI antibody (1:1000, Peninsula laboratories, Bachem, Bubendorf, Switzerland). For detection, horseradish peroxidase (HRP)-conjugated secondary antibody was used (1:15000, Zymed Laboratories, San Francisco, CA). Antibody-antigen complexes were visualized with ECL Plus (GE Healthcare, Chalfont St. Giles, UK). Detection was done with Storm 860

Imager and band densities were analyzed with ImagequantNT™ program (Molecular Dynamics, Sunnyvale, CA).

Real-time quantitative PCR

Total RNA was extracted from mouse tissues using RNeasy (QIAGEN) and cDNA was synthesized with TaqMan Reverse Transcription Reagents (Applied Biosystems). Genomic DNA was extracted by standard methods. Specific primers for DBI transcript were 5′GAA GCG CCT CAA GAC TCA GC3′ (forward) and 5′TTC AGC TTG TTC CAC GAG TCC3′ (reverse) and for *DBI* gene 5′CAC TCA GGC AAC CTA CAT C3′ (forward) and 5′GAA CAG CAT CTC TTC ATC AG3′ (reverse). Primers for 18 s RNA are described by Schmittgen and Zakrajsek (2000). PCR reactions were performed with ABI-PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA), in total volume of 30 µl. Reactions contained 5–10 ng of sample, 1× SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) and 9 pmol of DBI primers or 5 pmol of 18 s primers. All samples were done as duplicates in following conditions: 2 min at 50°C and 10 min at 95°C followed by 42 cycles of 15 s at 95°C and 1 min at 62°C. Each assay included a relative standard curve of tree serial dilutions of cDNA from sg mouse brain/genomic DNA, sample cDNA/DNA and no template controls. Results were calculated according to the manufactures' instructions.

Histological and morphological analysis

Mice were perfused intracardially with 20 ml of PBS and then 3×20 ml of 4% PFA. Brains were immerse-fixed in 4% PFA for 4 h and moved to PBS. Cryoprotection was carried out using 20% sucrose in PBS for 24 h. Brains were frozen in liquid nitrogen-isopentanol solution for 20 min. Brain slices of 16 µm were cut using cryotome. The whole brain was cut and every third slice was taken to staining.

Immunohistochemistry

DBI-immunoreactive neurons were stained utilizing the biotin-avidin technique. Briefly, the sections were treated with 1% hydrogen peroxide to remove endogenous peroxidase, and washed in potassium phosphate-buffered saline (KPBS), pH 7.4 before blocking in 10% normal goat serum (NGS) and 0.5% Triton X-100 in KPBS. Thereafter, sections were incubated for 3 days (+4°C) in DBI antibody (1:16000, Paesel-Lorei, Hanau, Germany) in 1% NGS, 0.5% Triton X-100 in KPBS. The sections were washed with KPBS containing 2% NGS before incubation for 1 h with

Table 4

Number of degenerating neurons in after KA injection

Brain area	Mouse group	Score statistics	
		N	Mean±SD
Granule	tg f	6	0.01±0.02
	sg f	6	0.10±0.05
	P value		0.030*
Hilus	tg f	6	0.31±0.21
	sg f	6	0.27±0.14
	P value		0.688
CA3c	tg f	6	0.37±0.06
	sg f	6	0.37±0.26
	P value		0.520
CA3b	tg f	6	0.27±0.11
	sg f	6	0.36±0.08
	P value		0.108
CA3a	tg f	6	1.32±0.44
	sg f	6	1.57±0.48
	P value		0.310
CA1	tg f	6	0.93±0.11
	sg f	6	1.60±0.10
	P value		0.631

Data are shown as mean score±SD.

biotinylated goat anti-rabbit immunoglobulin G (1:1500 Vector, Burlingame, CA). After washing in KPBS with 2% NGS, sections were incubated in avidin–biotin solution (Vectastain Elite ABC kit (Standard), Vector, Burlingame, CA) for 45 min and washed in KPBS with 2% NGS. Incubations in the secondary antibody and in the avidin–biotin solution were repeated. DBI antibody complex was visualized with 0.05% 3',3'-diaminobenzidine (DAB; Pierce Chemical, Rockford, IL), and 0.05% hydrogen peroxide in KPBS. Sections were mounted onto gelatin-coated slides, dried overnight at 37°C, and intensified with osmium tetroxide and thiocarbohydrazide (Lewis et al., 1986).

Cellular localization of DBI

To examine the cellular localization of DBI, double immunostaining with neuronal (neuronal nuclei, NeuN), astrocytic (glial fibrillary acidic protein, GFAP) was performed. In short, sections were rinsed in KPBS, blocked in 10% normal horse serum (NHS) and incubated for 3 days at 4°C in a primary antibody mix containing DBI antibody (1:1000, Peninsula Laboratories, Bachem, Bubendorf, Switzerland) and NeuN antibody (1:15000, Chemicon International, Temecula, CA) or GFAP antibody (1:2000, Roche, Mannheim, Germany). After washing sections were incubated overnight at 4°C with the secondary antibody mix containing Cy2-conjugated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA) and biotinylated horse anti-mouse IgG (1:200, BA-2000, Vector, Burlingame, CA) followed by an incubation of Cy5-conjugated streptavidin (2 µg/ml, Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were mounted on non-gelatinized slides with GelMount and dried overnight before analysis. Pictures were taken from granule cell layer and CA3 layer of hippocampus with Nikon Eclipse/ Ultra VIEW (Tokyo, Japan) confocal microscope.

Measurement of ventricle areas

The ventricle areas were measured from Nissl stained slides at 48-µm intervals using Stereo Investigator 2000 (MicroBrightField, Williston, VT) program in a system comprising of Olympus BX50 microscope linked to personal computer. Both ventricles from each section were measured blinded regarding to phenotype and the means were calculated.

High resolution magnetic resonance imaging (MRI)

Adult mice (tg $n=5$ and sg $n=5$) were anesthetized with an i.p. injection (50 µl/10 g body weight) of mixture of fentanyl-fluanisone and midazolame, and externally fixed to a custom-built animal holder for high resolution magnetic resonance imaging (MRI). Warm air was blown through the magnet bore during MRI. A s.m.i.s. console (Surrey Medical Imaging Systems, Guildford, UK) interfaced to a 9.4 T vertical magnet (Oxford Instruments, Oxford, UK) was used for MRI with a single loop surface coil (diameter 27 mm) in transmit/receive mode. Multi-slice T2-weighted images were acquired using a single-echo spin-echo method (time-to-repetition 3000 ms, time-to-echo 45 ms, 4 scans/line, field of view 25.6×12.8 mm² (transversal) or 25.6×25.6 mm² (coronal), matrix size 256×64 and slice thickness 0.75 mm).

Behavioral testing

Five wt and 6 tg male, 12 wt and 12 tg female mice (6 months old at the time of testing) were used for behavioral analysis. Behavioral test battery included the tests for exploratory activity (elevated plus maze, light–dark box, Y-maze, open field), nociception (hot plate), coordination (beam walking, rotarod), and learning and memory (fear conditioning, water maze). All tests were performed essentially as described by Voikar et al. (2004).

Fear conditioning (FC)

This is a form of classical conditioning where two memory components can be analyzed—association of unconditioned stimulus (foot shock, US)

with a particular compartment (contextual memory) and simple association of conditioned stimulus (tone, CS) with shock. The experiments were carried out employing a computer-controlled fear conditioning system (TSE, Bad Hamburg, Germany). Training was performed in a clear acrylic cage (35×20×20 cm) within a constantly illuminated (550 lx) fear conditioning box. A loudspeaker provided a constant, white background noise (68 dB) for 120 s followed by 10 kHz tone (CS, 75 dB, pulsed 5 Hz) for 30 s. The tone was terminated by a foot shock (US, 0.7 mA, 2 s, constant current) delivered through a grid floor. Two CS-US pairings were separated by a 30-s pause.

Contextual memory was tested 24 h after the training. The animals were returned to the conditioning box and total time of freezing (defined as an absence of any movements for more than 3 s) was measured by infrared light barriers scanned continuously with a frequency of 10 Hz. The CS was not used during this time. Memory for CS (tone) was tested 2 h later in a novel context. New context was a similarly sized acrylic box. The light intensity was reduced to 100 lx, the floor was plane (without shock grid) and the background colour was black (as opposed to white colour in training context). After 120 s of free exploration in novel context the CS was applied for additional 120 s and freezing was measured as above. In addition, the activity was registered in all phases of training and testing. Freezing behavior and activity were later corrected for the baseline measures (preconditioning for context test and new context for cue test) and expressed as a change from the respective baselines.

Water maze (WM)

The WM test was introduced for testing spatial learning and memory in rodents (Morris, 1981). The system used consisted of a black circular swimming pool (diameter 120 cm), escape platform (diameter 10 cm) submerged 0.5 cm under the water surface in the centre of one of four imaginary quadrants and computer interfaced video tracking system (EthoVision, Noldus, The Netherlands). The animals were released to swim in random positions facing the wall and the time to reach the escape platform was measured in every trial. Two training blocks consisting of three trials each were conducted daily. The interval between trials was about 5 min and between training blocks about 5 h. The platform remained in a constant location for 3 days (6 sessions) and was thereafter moved to the opposite quadrant for 2 days (4 sessions). The transfer tests were conducted approximately 18 h after the 4th, 6th and 10th training sessions. The spatial memory was estimated by the time spent in the zone around the platform (covering 6.25% of the total area of the water tank) and in corresponding zones of the three remaining quadrants. In addition, the swimming distance and the thigmotaxis were measured. Thigmotaxis was defined as the time spent swimming within the outermost ring of the water maze (10 cm from the wall).

Evaluation of synaptic transmission and induction of long-term potentiation (LTP)

Tg DBI mice ($n=17$) and their sg littermates ($n=18$), 5–8 months old, were used. Both females ($n=20$) and males ($n=15$) were investigated to see whether a sex difference causes changes in synaptic neurotransmission and/or plasticity. Mice were anesthetized with halothane and brains rapidly dissected. Slices were obtained from the middle third of hippocampi in both hemispheres and stored in a holding chamber at room temperature (~24°C). The holding chamber was continuously gassed with 95% O₂–5% CO₂. Individual hippocampal slices were transferred for recording into a submerged chamber held at 32±1°C with a bathing solution containing (in mM) NaCl 124, KCl 3, KH₂PO₄ 1.25, CaCl₂ 3.4, MgSO₄ 1.0, NaHCO₃ 26, D-glucose 10 and L-ascorbate 2. Slices were bubbled with 95% O₂–5% CO₂ to prevent calcium precipitation and perfused with a rate of 2.0 ml/min.

Electrophysiological recordings were made from the apical dendritic field of CA1 area in the hippocampal slice using a glass microelectrode filled with 2 M NaCl. Bipolar electrode made of twisted tungsten wire (50 µm) for stimulation was placed in the stratum radiatum of CA1 at a lateral distance of minimum 200 µm from the recording electrode. The stimulation intensity was adjusted to obtain about 50% of the maximal excitatory postsynaptic

potential (EPSP) amplitude and input was stimulated (0.1-ms pulse duration) every 30 s. If the baseline of EPSP responses did not stabilize, the experiment was discontinued. Paired pulse facilitation (PPF) was assessed by measuring the percent change of the second response relative to the first response to a pair of stimulation pulses separated by 75 ms. After a stable baseline period (20 min), LTP was elicited in the pathway by two applications of 10 bursts (inter burst interval 30 s) of high-frequency stimulation in a ‘theta burst’ pattern (TBS), i.e., 100 Hz trains of 4 pulses separated by 200 ms (Larson and Lynch, 1986; Pussinen and Sirvio, 1998). For maximal induction of LTP, the stimulation intensity was increased by setting the pulse duration to 0.3 ms. Monosynaptic field EPSPs were band-pass filtered from 0.1 Hz to 3 kHz and digitized at 10 kHz. Data were recorded and analyzed by microcomputer using the pCLAMP8.0 program (Axon Instruments, Foster City, CA).

Seizure induction with kainic acid

Animals, sexes and weights in the different treatment groups are summarized in Table 2. Kainate (KA) (in 0.9% NaCl, 26–30 mg/kg, Opika-1™ Kainic Acid, Ocean Produce International, Nova Scotia) was given i.p. One observer, blind to the genotype of the animals, determined the occurrence of behavioral seizures in mice visually for 4 h after KA injection. Time to the first behavioral manifestations of seizures, number and severity of behavioral seizures during the 4-h follow-up were scored according to a modified Racine's scale (Racine, 1972; Lahteinen et al., 2003).

Seizure induction with PTZ (Sigma, St. Luis, MO)

Eight sg and 8 tg male mice (5 months old, weight 27.0–38.5 g) were used for studying the seizure threshold with PTZ. PTZ was dissolved in 0.9% NaCl and 60 mg/kg was injected i.p. Two observers, blind to the genotype, monitored the animals for 1 h after the injection. Time to the first behavioral manifestations of seizures, number and severity of the seizures during the 1-h follow-up were recorded.

Histological evaluation after seizure induction

Mice injected with 28 mg/kg KA were perfused as described above 48 h after injection. Brain sections were made in the coronal plane (30 µm, one-in-five series). The amount of degenerating neurons after KA injection was evaluated using Fluoro-Jade B staining. After decreasing alcohol concentrations slides were bathed in a solution containing 0.06% potassium permanganate for 15 min, washed and incubated in Fluoro-Jade B solution containing 0.001% Fluoro-Jade stain in acetic acid. After washing, slides were dried, dehydrated in an alcohol series and xylene and mounted with DePeX. The amount of degenerating neurons in granule, hilus, CA3a, CA3b, CA3a and CA1 areas was visually evaluated as triplicates and scored. Evaluation started at the section where the suprapyramidal and infrapyramidal blades connected at the first time and continued for six following sections.

Statistical analysis of data

Results are calculated by Student's *t*-test or Mann–Whitney *U* test when appropriate. Significance of differences in mean values of synaptic responses and LTP was tested by analysis of variance (ANOVA, MANOVA). All statistical tests were done using SPSS 11.5 software or GrapPad InStat (GrapPad software Inc., San Diego, CA).

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