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Overexpression of γ-aminobutyric acid transporter subtype I leads to cognitive deterioration in transgenic mice[1]

MA Ying-Hua, ZHOU Xiao-Gang, DUAN Shu-Hui[2], HU Jia-Hua, LU Bu-Feng, YU Ying, MEI Zhen-Tong[2,3], FEI Jian[3], GUO Li-He[4] (*Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences; [2]Shanghai Institute of Physiology, Chinese Academy of Sciences, Shanghai 200031, China*)

KEY WORDS carrier proteins; gene expression; cognition; learning; memory; synapses; transgenic mice

ABSTRACT

AIM: To explore the physiological role of γ-aminobutyric acid transporter subtype I (GAT1) in cognition. **METHODS**: Transgenic mice were produced by pronuclei microinjection method. Integration of transgene was identified by Southern-blot and PCR analysis in various generations. Level of GAT1 mRNA in a variety of tissues was evaluated by semi-quantitative RT-PCR analysis. GAT1 protein was detected by immunofluorescence and histochemistry analysis. Associative learning capacity was analyzed by conditioned avoidance task. Memory retention was assessed by novel object recognition test. Morphology of synaptosomes was examined by electron microscope. **RESULTS**: Four independent founder mice bearing various copies of transgene were generated. GAT1 was evidently overexpressed at both mRNA and protein level in a variety of tissues from transgenic mice. In comparision with wild-type mice, transgenic mice exhibited significantly declined associative learning capacity ($P < 0.01$) and decreased memory retention ($P < 0.01$ in 1-h-retention, and $P < 0.05$ in 1-d-retention). In addition, the amount of asymmetric synapses in the brain of transgenic mice was reduced approximately by 24 %, relative to wild-type mice. **CONCLUSION**: Overexpression of GAT1 in mice results in cognitive deterioration, indicating that the alteration in the expression of γ-aminobutyric acid transporters is involved in the pathophysiological mechanism underlying some cognitive deficiencies.

[1] Project supported by the National Natural Science Foundation of China, № 39630140.
[3] Alternative correspondence to
Prof MEI Zhen-Tong. E-mail ztmei@server.shcnc.ac.cn
Dr FEI Jian. E-mail Fei@guomai.sh.cn
[4] Correspondence to Prof GUO Li-He.
Phn 86-21-6431-5030, ext 2069. Fax 86-21-6433-1090.
E-mail mhzhang@sunm.shcnc.ac.cn
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INTRODUCTION

γ-Aminobutyric acid (GABA) exists in the central nervous system (CNS) and functions as the predominant inhibitory neurotransmitter. As well known, an essential property of synaptic transmission is the rapid termination of neurotransmitter action, which is mostly achieved through the re-uptake of the transmitter by specific high-affinity transporters that are localized to pre-synaptic terminals or glial cell process. In addition, transporters play an important role in the regulation of the magnitude and duration of neurotransmitter's action[1]. Transporters are therefore key functional components of the neurotransmission in the nervous system. In the subfamily of GABA transporters, GABA transporter subtype I (GAT1) is the predominant neuronal transporter in the rodent brain, which was suggested by its pharmacological criteria and immunocytochemical localization[2]. Previous studies suggested that the damage of GABAergic transmission would result in amnesia and other memory deficiencies[3]. Since synaptic transmission mediated by GABA plays a key role in controlling neuronal activity and information processing in the mammalian cerebral cortex[4], it is possible that the GABA transporters are involved in the cognitive function. To test this hypothesis, we created the transgenic mice with murine GAT1 (mGAT1) overexpression and examined cognitive behaviors in such animals.

MATERIALS AND METHODS

Generation of transgenic mice A full length cDNA coding for mGAT1 that screened from λ phage murine brain cDNA library was cloned into the EcoR I and Apa I site of pcDNA3 (Fig 1a) under the control

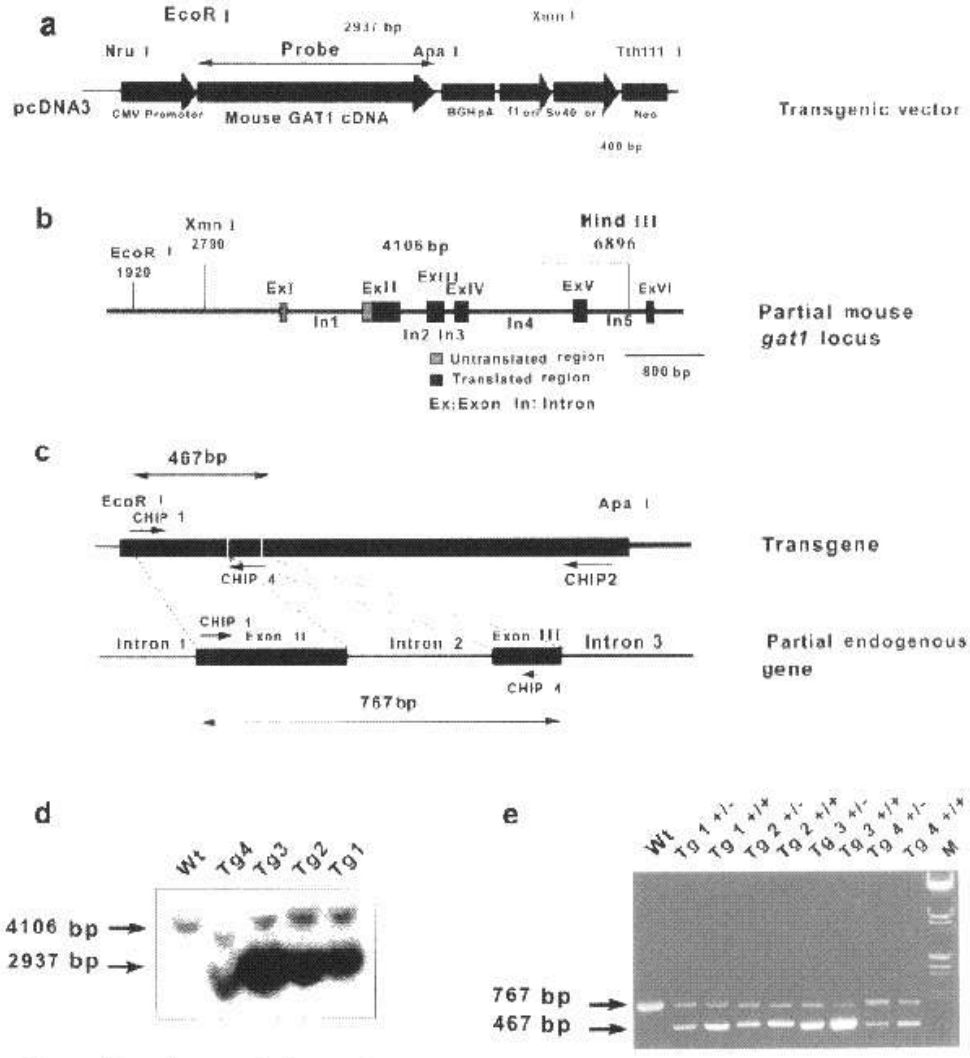

Fig 1. Construction and genotypic characterization of GAT1 transgenic mice. a) The construct of pcDNA3-GAT1 for the production of transgenic mice. b) The organization of partial endogenous mouse *gat1* locus. c) The schematic strategy of PCR analysis. Positions of primers (CHIP 1 and CHIP 4) for both transgenic and endogenous templates are indicated by single arrow. d) Southern-blot analysis of four independent founder mice (Tg 1–4). Wild-type mouse (Wt) was used as negative control for transgene and internal standard for endogene. e) PCR analysis of the progeny of four founder mice. Genotypes of homozygotes (+/+) and heterozygotes (+/−) raised from four independent transgenic lines are indicated respectively above each lane. M: λ/EcoR I+Hind III.

of human cytomegalovirus (HCMV) promoter/enhancer. This construct, linearized with Nru I and Tth111 I and subsequently gel-purified, was microinjected into the pronuclei of fertilized eggs of (C57BL/6J) F1 hybrid mice (Jakcson Laboratory, USA). Transgenic founders and their progenies were identified by polymerase chain reaction (PCR) and Southern-blot analysis.

Genotypic characterization of transgenic mice For Southern-blot analysis, 10 μg genomic DNA extracted from mice tail was digested with EcoR I, Xmn I, and Hind III, separated by electrophoresis on 0.7 % agarose gel, and transferred to a nylon membrane (Amersham Pharmacia Biotech, UK), followed by hybridization with a randomly ^{32}P-labeled mGAT1 full length cDNA probe. After hybridization, blots were washed and exposed against an X-ray film for 36 h and scanned with a

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PhosphorImager (Molecular Dynamics, USA).

For PCR analysis, the primers (CHIP 1: 5'ACCAAGCTTAGGCTGCAAAGCTGCTG3'; CHIP 4: 5'AGGCCTTTGAACATGGGCGCCAG3') were designed to match nucleotides from (−92) to (+375) of mGAT1 cDNA. With this primer pair, amplification was carried out as follows: 94 ℃ for 45 s, 64 ℃ for 45 s, and 72 ℃ for 60 s.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) Tissues were removed from adult wild-type and homozygous transgenic mice after cervical dislocation. Whole RNA was extracted with Trizol reagent (GIBCOBRL Life Technologies, USA) as detailed by the manufacturer's protocol, and identified by formaldehyde-electrophoresis. Before reverse transcription, RNA samples were thoroughly treated by incubating with RNA-Free DNase (5 U per μg RNA) for 45 min at 37 ℃. Using mGAT1-specific primers, PCR was carried out with conditions mentioned above.

GAPDH mRNA was co-detected with primers (5' end: 5' ACGACCCCTTCATTGACC 3'; 3' end: 5'AGACACCAGTAGACTCCACG 3') which matched at (+141) and (+344) of the murine GAPDH cDNA. Resultant GAPDH RT-PCR products were referred to quantify the expression level of mGAT1. RT-omitted RNA samples were directly amplified by PCR with 5-fold amounts of the corresponding templates, employed in the amplifications mentioned above, to demonstrate whether the amplified products were derived from mRNA or genomic DNA.

***In situ* immunofluorescent and immunohistochemical analysis** *In situ* immunofluorescent and immunohistochemical analysis of the expression of GAT1 in mice were respectively performed according to standard protocols.

Behavoral experiments Sex- and age-matched wild-type control mice and all independent transgenic lines were housed in groups in temperature- and humidity-controlled environment with a 12 h-light/12 h-dark rhythm. All behavioral experiments were performed with experimenter blind to the genotype of each mouse.

Conditioned avoidance task Behavioral training was carried out in a one way avoidance box which consists of two compartments. Before training, the mouse was put into the box for 15-min adaptation. During the training, in one compartment the mouse received electric foot shock and in the other one no electric shock was delivered. Light (40 W) was used as conditioned stimulus (cue), and the electric foot shock (4 − 15 V DC), as unconditioned stimulus, was delivered at the end of 5-s light stimulation. Total duration of light stimulation and paired electric shock was 10 s. After sufficient training (trials), the animals can run to the safe compartment within 5 s of light stimulation in order to avoid foot shock. This was considered as conditioned response. Nine conditioned responses out of ten trials was defined as the learning criterion. The number of trials needed for reaching the learning criterion was considered as the learning ability of the mouse.

Novel object recognition task Mice were individually placed into an open-field box (50 cm × 50 cm × 30 cm high) for 10-min adaptation before training. During training sessions, two objects were placed into the open field and the animal was allowed to explore for 6 min. The time spent in exploring each object was recorded. During retention tests, the animal was placed back into the same box, in which one of the familiar objects used during training was replaced by a novel object, and allowed to explore freely for 6 min. A preference index, a ratio of the amount of time spent in exploring anyone of the two objects (object 1 or object 2 in training session) or the novel one (object 3 in retention session) over the total time spent in exploring both objects, was used to measure recognition memory.

Electron microscopy examination of synaptosomes Adult transgenic and wild-type mice were killed by decapitation after anesthesia. The forebrain regions were rapidly removed and then homogenized in sucrose 0.32 mol·L^{-1}. Crude synaptosomes were prepared as detailed elsewhere[5] and then processed according to Hajos *et al*[6] for electron microscopy analysis. Ultrathin sections were cut with Reichert ultramicrotome and double stained with uranyl acetate and lead citrate. Sections were examined and photographed under JEM100 B transmission electron microscope. The amount of the asymmetric synapses on each section was counted with 3 regions randomly selected and photographed under the same magnitude of amplification. Twelve sections from 4 mice were measured for wild-type and transgenic mice, respectively.

Statistical analysis All values were expressed as $\bar{x} \pm s$. The significant difference between each group in various experiments was analyzed by t-test or Dunnett's test.

RESULTS

Genotypic characterization of transgenic

mice Linearized DNA construct containing the full length mGAT1 cDNA under the control of cytomegalovirus promoter/enhancer (CMV) was introduced into the C57BL/6J mouse germ line by the microinjection method[7]. Four independent founders from 28 newborns were identified by PCR analysis (data not shown) and further determined by Southern-blot analysis of mouse tail genomic DNA restricted with EcoR I/Xmn I/Hind III (Fig 1d). Hybridization with the ^{32}P-labeled full length mGAT1 cDNA probe (Fig 1a) yielded two fragments for endogenous and transgenic gene, respectively (Fig 1d), which correspond to the expected size (Fig 1a, b). The relative numbers of transgene copies of the four transgenic strains, estimated by comparing the signal intensity of transgenic fragment to that of endogenous fragment with a PhosphorImager, was 4, 5, 8 - 10, and 2, respectively.

Founders were bred with wild-type mice to generate allelic heterozygotes, which were subsequently inbred and homozygous offsprings were born at the predicted Mendelian ratio. All progenies were characterized by PCR analysis. Consistent with the predicted size (Fig 1c), the endogenous gene appeared a 767-bp band, whereas the transgene generated a 467-bp band (Fig 1e). Homozygotes or heterozygotes raised from a same founder could be discriminated by respectively comparing the signal intensity of the fragment for transgene to that for endogene. The genotypes of transgenic mice were further verified by the subsequent identification of seven generations. All transgenic mice were viable and developed grossly normally, but with increased body weight and relatively reduced pregnancy efficiency[8].

Characterization of the expression level of GAT1 in transgenic mice The expression level of GAT1 in the fourth generation of two independent transgenic lines was assessed by semi-quantitative RT-PCR analysis. Employing primers designed for mGAT1, a 467-bp specific fragment was obtained, subsequently convinced by sequencing analysis (data not shown), from a variety of tissues except liver in both transgenic lines (Fig 2a). Contrasting sharply with transgenic mice did, no products could be achieved from the kidney and the heart of wild-type controls (Fig 2a). By normalization to the GAPDH mRNA level (Fig 2b), quantitative analysis with PhosphorImager revealed that both independent transgenic lines apparently exhibited increased expression of GAT1, with slightly varying level respectively, in the brain, spleen and testis, where the endogenous gene was expressed. Moreover, no specific PCR product from the reverse transcription-omitted RNA samples could be observed over background (Fig 2c), which convinced that the amplification of GAT1 was derived from mRNA rather than genomic DNA.

To confirm the expression of GAT1 in the kidney of transgenic mice but not in that of wild-type controls at protein level, we performed *in situ* immunoflurocent detection with mGAT1 antibody directed to the N-terminal of GAT1 ($mGAT1_{1-50}$)[9]. Indeed, GAT1-immunoreactivity localized to the glomerulus of the kidney could only be observed in transgenic mice but not in wild-type controls (Fig 3A), which was consistent with the results of RT-PCR.

Furthermore, immunohistochemical examination on the CA1 and CA3 field was carried out to evaluate the expression level of GAT1 in hippocampus, which is essential for learning and memory. PhosphorImager analysis revealed that transgenic mice exhibited apparently higher intensity of GAT1-immunoreactivity, localized to the soma, axon fibers, and numerous puncta structures, than did wild-type controls (Fig 3B). It indicated the increased expression of GAT1 in the hippocampus of transgenic mice. In addition, GAT1-immunoreactivity was prevented in sections incubated in anti-mGAT1 antibodies preabsorbed with mGAT1 N-terminal peptide (data not shown), which authenticated the specificity of the immunostaining of mGAT1. With all above results, we concluded that we had generated the transgenic mice with GAT1 overexpression.

Behavior analysis of transgenic mice in recognition tasks To explore whether the recognitive capacity was influenced in transgenic mice with GAT1 overexpression, preliminary behavioral experiments were conducted. In view of the opinion that GABAergic transmission is involved in the control of conditioned behavior[10], we first employed conditioned one-way avoidance task to assess the learning ability of both young (1-month-old) and adult (4-month-old) transgenic mice. The number of trials needed to reach the learning criterion was significantly increased in either young or adult transgenic mice ($P < 0.01$, Fig 4). This result indicated that the associative learning ability of transgenic mice was remarkably declined compared with that of wild-type controls. Moreover, the poor performance of young (1-month-old) transgenic mice suggested that the learning impairment occurred in the early developmental stage. No significant difference in the trial numbers, required for correct conditioned response, between young and adult transgenic mice was observed, which was sharply in contrast to that did wild-type controls. It meant that the

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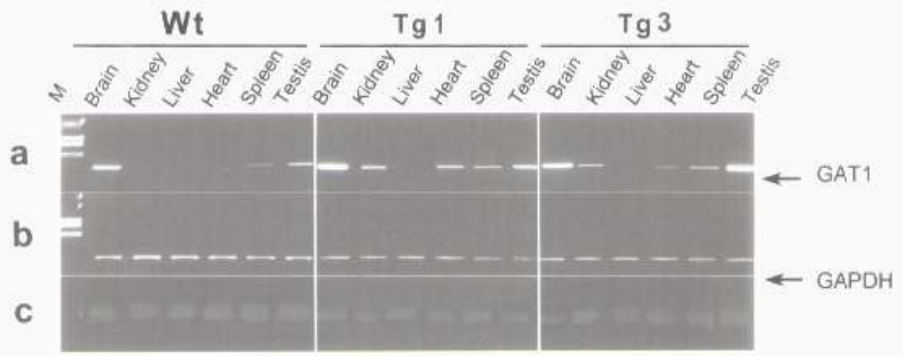

Fig 2. Semi-quantitative RT-PCR analysis of the expression of GAT1 in a variety of tissues from two independent transgenic lines (Tg 1 and Tg 3) and wild-type controls (Wt). a) Indicating the amplification of GAT1 mRNA with RT-conducted RNA samples using GAT1-specific primers (CHIP 1 and CHIP 4). b) Indicating the amplification of GAPDH mRNA which was served as an internal standard. c) Indicating the direct amplification with RT-omitted RNA samples using the same primers employed in "a".

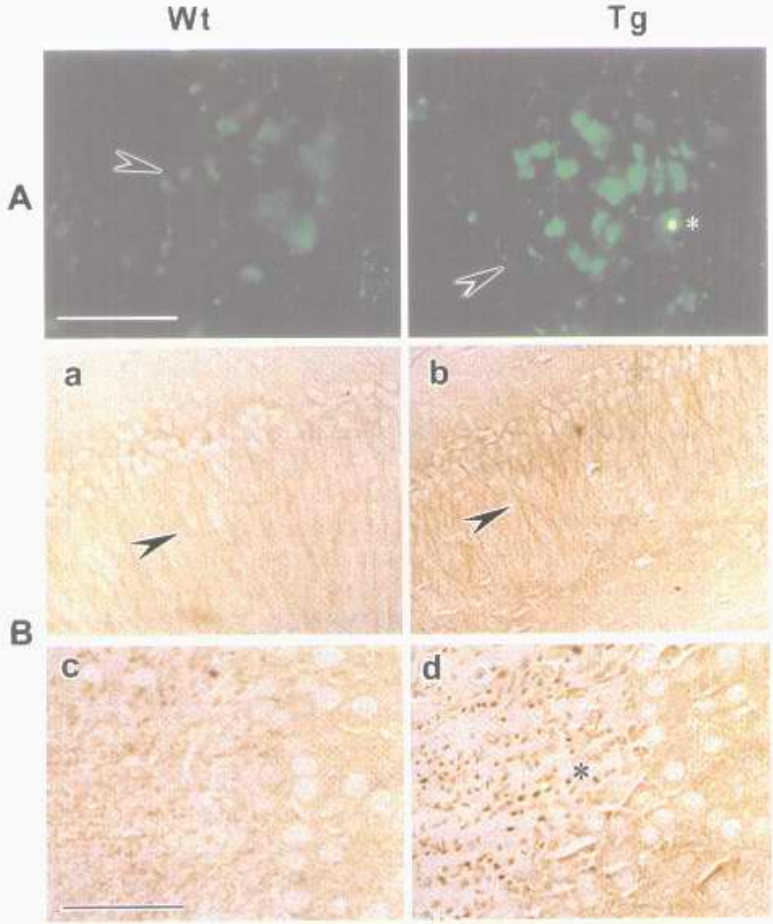

Fig 3. A) Immunoflorescent detection of the GAT1-immunoreactivity in the kidney from both wild-type (Wt) and transgenic mouse (Tg). Arrowhead points to the glomerulus. Asterisk indicates the non-specific immunostain. Scale bar represents 100 μm. B) Immunohistochemical analysis of the GAT1-immunoreactivity in the CA1 (a, b) and CA3 (c, d) field of hippocampus from both wild-type (left column) and transgenic mice (right column). Arrow points to the axon fibers. Asterisk indicates the punctate structures with high intensity of immunoreactivity. Scale bar represents 100 μm in "a" and "b" and 200 μm in "c" and "d", respectively.

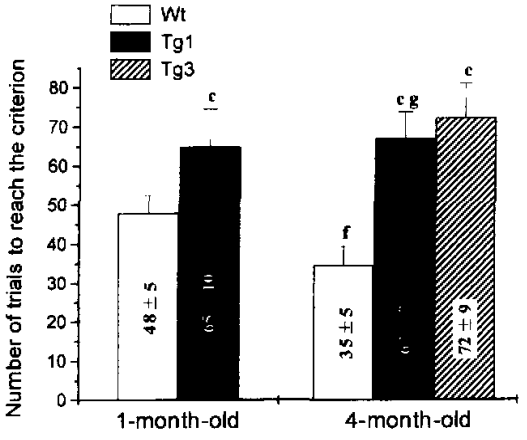

Fig 4. Conditioned avoidance task revealed impaired learning ability in young (1-month-old) and adult (4-month-old) transgenic mice raised from two independent lines (Tg 1 and Tg 3). n = 11 wild-type mice (Wt) and 10 Tg 1 mice in both age-group, respectively; n = 9 Tg 3 mice in 4-month-old group. $\bar{x} \pm s$. $^cP < 0.01$ *vs* age-matched Wt mice. $^fP < 0.01$ *vs* 1-month-old Wt mice. $^gP > 0.05$ *vs* 1-month-old Tg 1 mice.

learning capacity was not developed with aging in transgenic mice.

We further employed the novel object recognition task to measure visual recognition memory, which is evolutionarily conserved in species including humans and rodents and requires the participation of hippocampus[11-13]. During training, there was no significant difference in the exploratory preference (Fig 5a), indicating that both types of mice have the same preference to explore the objects. During the retention test (Fig 5b), one of the familiar objects used in the training session was replaced with a third novel object, and animals were allowed to explore for 6 min freely. Both transgenic lines exhibited obviously reduced preference towards the novel object than did the wild-type mice at either 1-h- or 1-d-retention test. This indicated that the transgenic mice were poor to retain the memory of the old object. A *post hoc* analysis using Dunnett's test revealed a significant difference between wild-type and either transgenic line at the 1-h- ($P < 0.01$) or 1-d-retention test ($P < 0.05$), but not between the two transgenic lines. The impaired memory was therefore independent of the transgene integration locus.

Morphological examination of the synaptosomes from transgenic mice To investigate whether

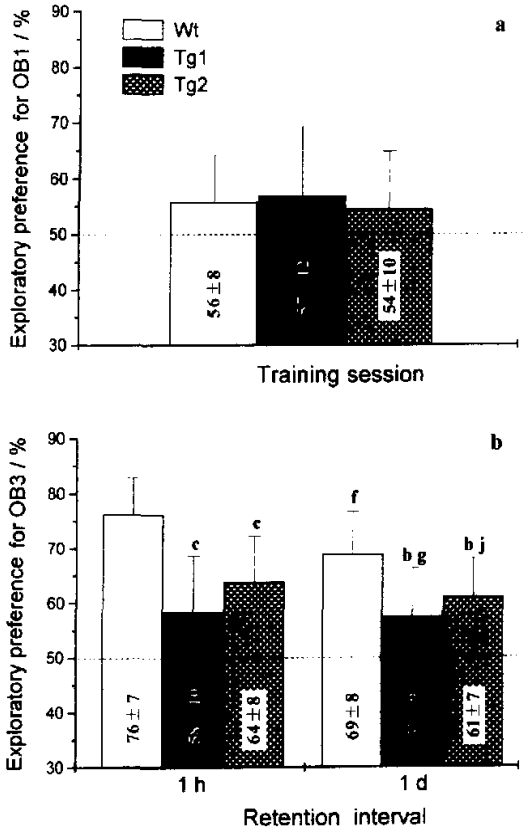

Fig 5. Impaired novel object recognition memory in transgenic mice. a) Exploratory preference in the training session was indistinguishable between wild-type mice (Wt) and two independent transgenic lines (Tg 1 and Tg 2). b) Significantly Reduced exploratory preference in transgenic mice in 1-h- and 1-d-memory retention test. Dotted line represents preference at chance 50 %. OB indicates objects employed in this experiment. n = 12 Wt mice, 11 Tg 1 mice, 9 Tg 2 mice in training session and each retention interval test. $x \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* Wt mice in each retention interval test. $^fP < 0.01$ *vs* Wt mice in 1-h retention test. $^gP > 0.05$ *vs* Tg 1 mice in 1-h retention test. $^jP > 0.05$ *vs* Tg 2 mice in 1-h retention test.

the impaired cognitive capacity of transgenic mice correlates with the alteration in the synaptic morphology, we performed electron microscopic examination on the synaptosomes, which were prepared from the adult (4-month-old) forebrain regions such as hippocampus and cortex. Interestingly, the amount of the asymmetric synapses featuring postsynaptic thickenings was reduced approximately by 24 % in transgenic mice compared with that in wild-type controls (Fig 6). Studies previously suggested that

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GAT1 correlates with the brain maturation[34], and all GAT1-positive axon terminals form symmetric synapses[35]. Therefore, this finding might indicate that the normal process of the formation of asymmetric synapses in the brain of transgenic mice was possibly disrupted by the overexpression of GAT1. It also suggested that GAT1 may be involved in some aspects of the process of synapse plasticity, which is considered as the basis of learning and memory.

DISCUSSION

Transgenic mice overexpressing GAT1 were generated. Authentically, two independent lines analyzed a little differently exhibited increased expression of GAT1 in a variety of examined tissues except liver compared with that did wild type controls. This pattern of transgene expression in transgenic mice was consistent with that observed in other transgenic lines containing CMV promoter driver constructs detailed previously[16]. Furthermore, apparently higher intensity of GAT1-immunoreactivity associated with soma and axon of pyramid neurons in CA1 field, also with punctate structures in CA3 field, which appeared to be axon fibers, indicated the overexpression of GAT1 in the hippocampus of transgenic mice. In addition, up-regulation of the activity of GABA transport in transgenic mice, demonstrated by synaptosomal uptake analysis (unpublished data), indicated that the overexpression of GAT1 was functionally significant.

Although no gross developmental abnormalities were observed, the transgenic mice significantly exhibited a series of impairments in cognition which include reduced associative learning ability and declined memory retention. The experiment of conditioned avoidance task indicated that the cognitive deterioration might initiate in the early developmental stage in transgenic mice. Failure to increase learning ability with developing in transgenic mice (Fig 4) suggested that the maturation of the brain may be disturbed by the abnormally increased expression of GAT1, which is potentially supported by the opinion that GABA transporters may be involved in the maturation of GABAergic inhibitory system in the brain[11]. In the novel object recognition task, to our surprise, two independent transgenic lines performed similarly in both 1-h- and 1-d-retention test, respectively (Fig 5b). In contrast to transgenic mice, wild-type controls meaningfully exhibited memory reduction in 1-d-retention test ($P < 0.01$), relative to 1-h-retention test. It indicated that transgenic mice probably possessed alternative mechanism of memory processing. Finally, it is noteworthy that the impairments in cognition occurre in all independent transgenic lines analyzed, therefore, the transgenic phenotype should not result from an insertional mutation.

Another interesting finding of this study was that the transgenic mice contained apparently reduced asymmetric synapses in the brain, relative to wild-type controls. It was previously reported that chronic treatment with GABA can induce the formation of free postsynaptic thickenings and promote synapse formation with foreign nerves implanted in the rat's superior cervical ganglion[17]. In

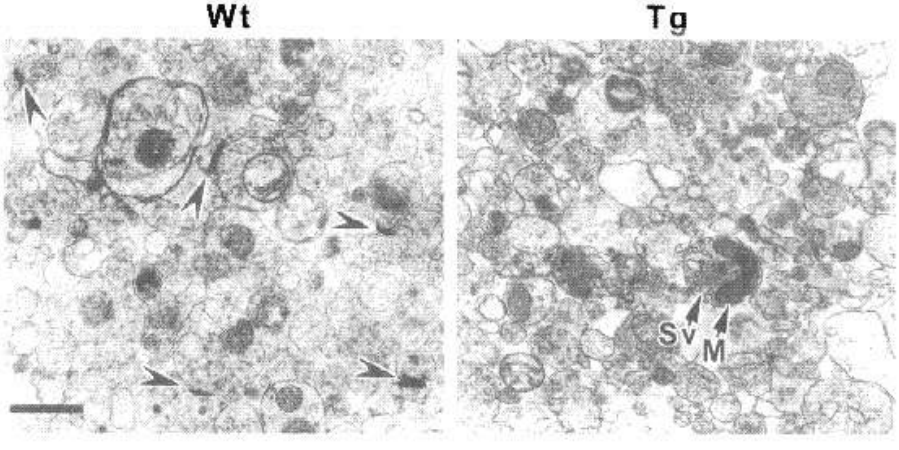

Fig 6. Electron microscopic appearance of synaptosomes. Transgenic mice (Tg) contain fewer (approximately 24 %) asymmetric synapses (indicated by arrowhead) than wild-type mice did (Wt). Mitochondria (M) and synaptic vesicles (Sv) were indicated in representative sections. Scale bar represents 1 μm. $n = 12$ sections from 4 wild-type and transgenic mice, respectively (3 regions randomly photographed were counted on each section).

culture, GABA seems to stimulate early events of synaptogenesis in murine neuroblastoma cells[18]. In view of theses above facts, it was probable that the overexpression of GAT1 might disrupt the formation of asymmetric synapses by attenuating the extracellular level of GABA. Additional studies should be required to explore the mechanism underlying this phenomenon and its potential correlation with learning and memory. Immediately, the extracellular concentration of GABA in the brain is needed to be fully characterized in transgenic mice.

In conclusion, our results demonstrated that overexpression of GAT1 in transgenic mice resulted in profound impairments in learning and memory. It strongly suggested that the alteration in GABA transporters may be a contributing etiological factor for certain types of cognitive deficiencies.

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过量表达 γ-氨基丁酸转运蛋白亚型 I 导致转基因小鼠认知衰退[1]

马映华，周筱刚，段书惠[2]，胡佳华，卢步峰，余 莹，梅镇彤[2,3]，费 俭[3]，郭礼和[4]
（中国科学院上海生命科学研究院生物化学和细胞生物学研究所；[2]中国科学院上海生理研究所，上海 200031，中国）

关键词 载体蛋白类；基因表达；认知；学习；记忆；突触；转基因小鼠

目的：研究 γ-氨基丁酸转运蛋白亚型 I（GAT1）在认知中的生理功能．**方法**：原核显微注射法产生转基因小鼠．Southern-blot 和 PCR 鉴定转基因的整合；半定量 RT-PCR 分析 GAT1 mRNA 水平；免疫荧光和免疫组化检测 GAT1 蛋白质表达．条件性回避行为分析联合型学习能力；新异物体识别行为分析记忆存储能力．电镜检查突触体形态．**结果**：获得 4 只含不同拷贝数转基因的建立者小鼠．GAT1 在 mRNA和蛋白质水平都呈过量表达．与野生型小鼠相比，转基因鼠联合型学习能力显著下降，视觉记忆显著减弱．另外，转基因鼠不对称突触数量减少约 24 %．**结论**：在小鼠中过量表达 GAT1 可导致认知衰退，表明 γ-氨基丁酸转运蛋白表达的改变与某些类型认知缺陷的病理机制有关．

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