γ-aminobutyric acid transporter-1 is involved in anxiety-like behaviors and cognitive function in knockout mice

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Abstract. The aim of the present study was to investigate the effect of γ-aminobutyric acid transporter-1 (GAT-1) on the anxiety-like behaviors and cognitive function in knockout mice. In total, 20 adult male mice were divided into two groups, namely the GAT-1 knockout (GAT-1$^{-/-}$) and wild-type (WT) groups. The open field test, elevated 0-maze (EZM) and Morris water maze were used to evaluate changes in anxiety-like behaviors and cognitive function. Compared with the WT mice, GAT-1$^{-/-}$ mice made more entries and spent a longer time within the central area, traveling a greater distance, during the open field test ($P<0.05$). The EZM revealed that GAT-1$^{-/-}$ mice spent more time in the open sectors and made more total entries when compared with the WT mice ($P<0.01$). Observations from the two tests indicated reduced anxiety-like behaviors in the GAT-1$^{-/-}$ mice. During the learning session using a Morris water maze, the latency to find the platform was significantly longer in the GAT-1$^{-/-}$ mice when compared with the WT mice ($P<0.01$). In addition, during the probe test, the GAT-1$^{-/-}$ mice spent less time in the target quadrant and more time in the opposite quadrant when compared with the WT mice ($P<0.01$); thus, the cognitive function in the GAT-1$^{-/-}$ mice was impaired. Therefore, the results demonstrated that the anxiety-like behaviors were reduced and cognitive function was impaired in GAT-1 knockout mice, indicating that GAT-1 is involved in anxiety and cognitive functions.

Introduction

γ-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter that activates $GABA_A$, $GABA_B$ and $GABA_C$ receptors in the mammalian brain (1,2). The inhibitory synaptic transmission is decreased or terminated by the reuptake of the released GABA in the synaptic cleft through GABA transporters, which is considered to be the key action of the current termination. GABA transporters are, therefore, involved in maintaining a low extracellular GABA concentration throughout the brain, preventing the tonic phenomenon caused by excessive activation of synaptic and extrasynaptic receptors (2).

GAT is highly expressed in the vesicle membrane, the presynaptic membrane and the glial cell membranes, and belongs to a family of electrogenic sodium-dependent transporters (3). Four GABA transporters (GAT-1-4) have been identified and cloned (4,5). The affinity of the receptors for GABA is as follows: GAT-1>GAT-3>GAT-2>GAT-4 (6); thus, GAT-1 has the largest ability to uptake GABA in the brain (5). GAT-1 is particularly abundant in areas rich in GABAergic neurons, such as the cerebellum, hippocampus, neocortex and retina (7). GAT-1 is primarily responsible for the removal of GABA from the synaptic cleft and the termination of GABAergic neurotransmission; therefore, the transporter plays an important role in the metabolism of GABA (8).

Increasing evidence has demonstrated that the GABA system is important in the pathogenesis of anxiety (9,10). In humans and animals, stimulation of GABA receptors generally produces anxiolytic activity, while antagonists produce anxiogenic-like effects (11). The GABA system is also known to be involved in the modulation of memory and learning (12,13); however, the underlying mechanisms are yet to be fully elucidated. In the present study, GAT-1 knockout (GAT-1$^{-/-}$) mice were used as a model. Three types of behavioral tests, namely the open field test, elevated 0-maze (EZM) and Morris water maze, were used to evaluate anxiety-like behaviors and cognitive function in the GAT-1$^{-/-}$ and wild-type (WT) mice.

Materials and methods

Animals. Experimental protocols and the use of animals were performed in compliance with the Guidelines for Animal Experiments of the Chinese Academy of Medical Sciences (Beijing, China) and with approval from the Ethics Committee for Animal Care at Jinshan Hospital (Shanghai, China). In total, 20 adult male GAT-1$^{-/-}$ and WT mice were obtained from

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Shanghai South Biomodel Organism Co., Ltd. (Shanghai, China) and housed in the animal center of Jinshan Hospital. The animals were maintained under a 12-h light/dark cycle at 22˚C and 50% humidity. The animal rooms were kept neat and uncluttered. The drinking water was autoclaved and changed everyday, and the animal cages were disinfected by ultraviolet light. The padding used in the cages was also changed everyday. Food and water were available *ad libitum,* with the exception of during the tests. The mice (age, 6-8 weeks) were divided into two groups (n=10): GAT-1$^{-/-}$ and WT.

Open-field test. The open-field test is widely used to test anxiety-like behavior and activity in animals (14). Mice have an innate tendency to escape bright, open new surroundings. The open-field test can cause anxiety-like behavior; thus, the test is applicable to the assessment of motor behavior caused by anxiety. The open field (DuoYi animal behavior analysis system; Shanghai Mobile Datum Co., Shanghai, China) was a square arena (50 cm^3) with white plastic walls and floor (ABS engineering plastic; Shanghai Mobile Datum Co., Shanghai, China). The mice were placed in the center of the box and allowed to freely explore for a 5-min period. The mice were recorded using a camera (Shanghai Mobile Datum Co.,) fixed above the floor, which was analyzed with a video tracking system (Shanghai Mobile Datum Co.) that divided the arena into 'margin' and 'center' fields. The center field was defined as the central 25% area of the open field. The software (Shanghai Mobile Datum Co.) automatically recorded a mice motion curve, identifying the movement and stationary state of the mice. The arenas were cleaned with a 70% alcohol solution between trials to remove excrement and odor.

EZM. The exploratory drive of mice and their natural avoidance of heights and open spaces were used in the EZM to investigate the anxiety-like behavior of the mice (14). The EZM is modelled on the elevated-plus-maze. The advantage of the EZM is that it removes the ambiguous central square of the traditional elevated-plus-maze. The EZM (Shanghai Mobile Datum Co.) consisted of a circular platform (46 cm in outer diameter, 5.5 cm in runway width) that was elevated 40 cm above the floor. On the top of the platform, there were two open and two enclosed segments. The closed segments were enclosed by walls extending 20 cm above the surface of the platform. Each test started by placing the mouse in any closed sector, and the test session lasted for 5 min. Performance was recorded using a video-camera placed above the EZM. The video tracking software recorded the path moved, the percentage of time spent in the open and closed segments, and the number of open and closed segment entries. The EZM was cleaned with water between trials.

Morris water maze. The water maze was used to measure spatial learning and memory ability (15,16). The apparatus (Shanghai Mobile Datum Co.) was a circular swimming pool (100 cm in diameter, 50 cm high) with black plastic walls and floor (ABS engineering). The swimming pool was filled with water maintained at 24-26˚C to a depth of 30 cm. Water was added with milk powder to enable clear observations of the black mice and record their movement curve. The pool was divided into four equal quadrants by four entry points marked on the pool wall and a white escape platform was set in the center of the target quadrant (1 cm below the water level). Each quadrant was marked with a different shape in order to provide visual clues to aid the mice in finding the escape platform. The position of the platform was fixed throughout the place navigation test. The platform was the only escape route for the mice in the water; thus, the mice were required to search for the hidden underwater platform. This task consisted of place navigation tests four times a day for five consecutive days, with intertrial intervals of 15-20 min, followed by probe trials on the sixth day. In each trial, a mouse was released into the water, facing the pool wall, from one of the quadrants with the exception of the target quadrant. The mice were allowed to swim for a maximum of 60 sec until they found the platform. If the mouse failed to find the platform in 60 sec, the mouse was gently placed on the platform and allowed to stay on it for 10 sec prior to the next trial. On the probe trial day, the platform was removed and each mouse was released into the pool from the same position. The swimming paths of the mice were recorded for 60 sec and monitored by a camera mounted above the center of the pool. Following the trial, the mice were placed in clean padding and allowed to warm up and dry. The room was maintained at 22-24˚C and the water in the pool was changed everyday.

Data collection from the open-field test. Rodents prefer to move around the periphery of an apparatus than explore the central area when they are placed in a novel environment (17). This feature can protect animals from the invasion of outsiders. The time spent in and the number of visits to the central area of the open field is considered to be inversely correlated with the level of anxiety-like behavior of mice, while the movement distance and the kinematic velocity reflect the motility and active degree (18).

The following parameters were assessed: Total distance moved, velocity, the rest time during observation periods and time spent in the central area, number of visits to the central area and the distance traveled in the central area.

Data collection of EZM. During an EZM, based on their natural avoidance of heights and open spaces, mice usually avoid the two open arms and spend the majority of time in the two closed arms, while the search for novel, open environment drives them into the open areas (19). The time spent in the open spaces and the total number of open arm entries and closed arm entries are inversely correlated with the level of anxiety-like behaviors (20). In addition, the distance traveled in the maze reflects the motility and the degree of activity.

The following parameters were assessed in the EZM test: The number of total entries to the open arms and closed arms, the proportion of time spent in the open arms and the distance traveled in the maze.

Data collection of Morris water maze. Although mice are natural swimmers, they dislike the state of being in the water. Furthermore, swimming is physically exhausting, and mice instinctively seek the rest area in the water. This behavior involves a complex process of memory, including collecting visual information associated with the spatial orientation, dealing with, sorting, memorizing and strengthening the information, with the purpose of finding the hidden platform in the water (21) and finally escaping from the water. In the training

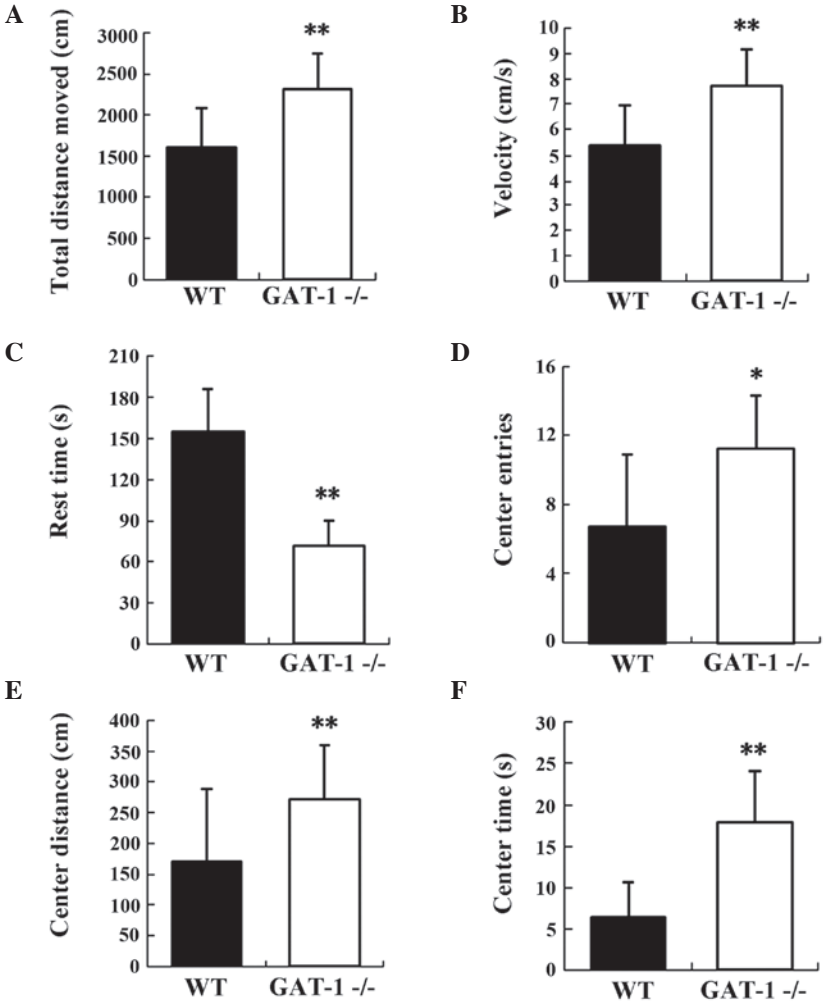

Figure 1. Performance of GAT-1$^{-/-}$ and WT mice in the open-field test. (A) Total distance traveled; (B) kinematic velocity; (C) rest time; (D) entries into the central area; (E) distance traveled in the central area; and (F) time spent in the central area. *P<0.05 and **P<0.01, vs. WT group (n=10 per group). WT, wild-type; GAT, γ-aminobutyric acid transporter.

session, mice with a shorter escape latency are considered to have stronger abilities of spatial learning and memory. While in the probe trial, mice that spent a longer time in the target quadrant had a more accurate location and spatial memory (15).

The following parameters were assessed during the Morris water maze: Average escape latency in the navigation test, the proportion of time spent in each quadrant and the swimming trace in the probe trial.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Differences between the two groups in the navigation test in the Morris water maze were compared using repeated-measures one-way analysis of variance (ANOVA). Other data comparisons were analyzed using an independent sample t-test. P<0.05 was considered to indicate a statistically significant difference in all the statistical evaluations. Statistical analyses were performed using SPSS 19.0 statistical software (IBM SPSS, Inc., Armonk, NY, USA).

Results

Assessment of the open-field test. In the open-field test, GAT-1$^{-/-}$ mice traveled greater distances compared with the WT mice (GAT-1$^{-/-}$, 2,312.98±439.58 cm; WT, 1,607.78±476.26 cm; P<0.01), and exhibited enhanced kinematic velocity (GAT-1$^{-/-}$, 7.70±1.46 cm/sec; WT, 5.36±1.59 cm/sec; P<0.01), with a significant reduction in rest time (GAT-1$^{-/-}$, 71.97±18.42 sec; WT, 155.30±30.32 sec; P<0.01). The GAT-1$^{-/-}$ mice manifested hyperactivity and enhanced motility compared with the WT mice. In addition, the GAT-1$^{-/-}$ mice spent more time in the central area (GAT-1$^{-/-}$, 17.87±6.16 sec; WT, 6.43±4.20 sec; P<0.01) and made more entries into the central area when compared with the WT mice (GAT-1$^{-/-}$, 11.22±3.0; WT, 6.70±4.22; P<0.05). GAT-1$^{-/-}$ mice also showed a significant increase in the distance traveled in the central area (GAT-1$^{-/-}$, 272.36±87.09 cm; WT, 170.39±117.68 cm; P<0.01). These parameters are inversely correlated with the level of anxiety-related proneness, indicating that GAT-1$^{-/-}$ mice showed decreased anxiety-like behaviors in comparison with the WT mice (Fig. 1).

Assessment of the EZM test. GAT-1$^{-/-}$ mice manifested hyperactivity and enhanced motility in the EZM test, as compared with the WT mice, and traveled greater distances (GAT-1$^{-/-}$, 14,097.96±2,775.40 cm; WT, 3,356.12±968.37 cm; P<0.05). The results revealed that GAT-1$^{-/-}$ mice had a significantly higher total number of entries into the open and closed sectors

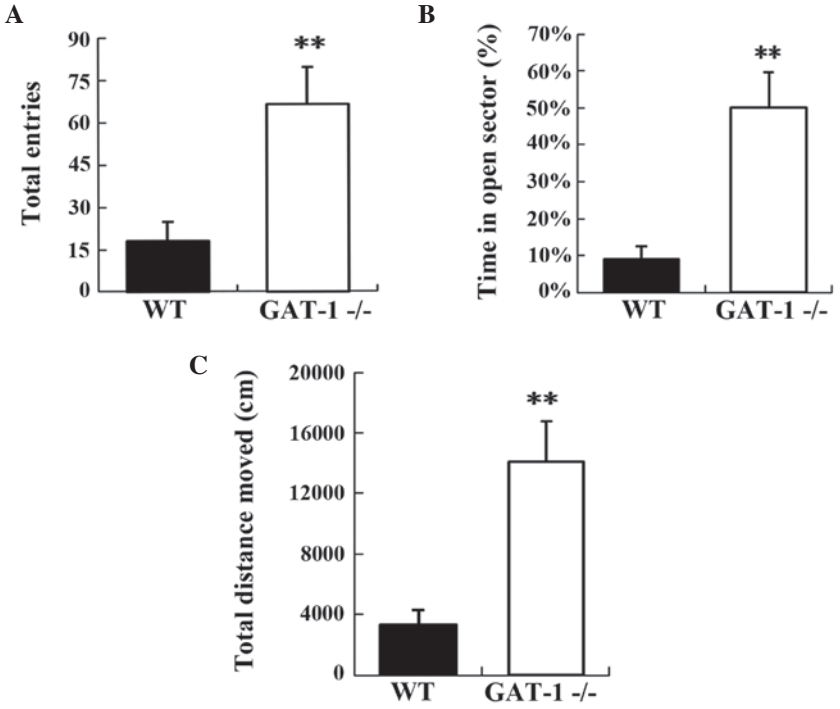

Figure 2. Performance of GAT-1$^{-/-}$ and WT mice in the enhanced 0-maze test. (A) Total number of entries into the open and closed sectors; (B) percentage of entries into the open sectors compared with the total entries; and (C) percentage of time spent in the open sectors. **P<0.01, vs. WT group (n=10 per group). WT, wild type; GAT, γ-aminobutyric acid transporter.

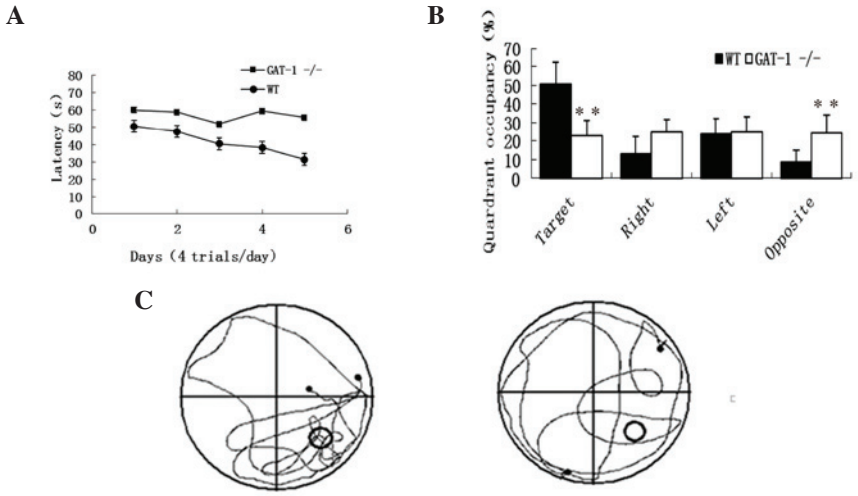

Figure 3. GAT-1$^{-/-}$ mice exhibited impaired learning and memory in the Morris water maze test. (A) Latency to find the platform during the learning sessions; (B) time spent in the target quadrant or opposite quadrant during the probe test; and (C) typical swimming traces during the probe test of the WT and GAT-1$^{-/-}$ mice. **P<0.01, vs. WT (n=10 per group). WT, wild type; GAT, γ-aminobutyric acid transporter.

compared with the WT mice (GAT-1$^{-/-}$, 66.78±13.07; WT, 18.00±6.83; P<0.01). In addition, the GAT-1$^{-/-}$ mice exhibited an increased percentage of time spent in the open sectors when compared with the WT mice (GAT-1$^{-/-}$, 50.00±0.097%; WT, 9.00±0.036%, P<0.01). Therefore, the results confirmed that GAT-1$^{-/-}$ mice demonstrated reduced anxiety-like behaviors (Fig. 2).

Assessment of the Morris water maze test. During the learning session, repeated-measures ANOVA indicated a significant difference between the WT and GAT-1$^{-/-}$ mice (F=14.48, P=0.001), with GAT-1$^{-/-}$ mice exhibiting significantly longer latencies compared with the WT mice (F=3.23, P<0.05). Evidently, the GAT-1$^{-/-}$ mice learned at a slower pace in comparison to the WT mice. With regard to the GAT-1$^{-/-}$ mice, the time spent between the four quadrants during the probe test was not significantly different, while the WT mice spent 50.7% of the total time on the target quadrant and only 8.82% on the opposite quadrant. Compared with the WT mice, GAT-1$^{-/-}$ mice spent less time in the target quadrant (P<0.01).

These results confirmed that GAT-1$^{-/-}$ mice exhibited impaired spatial learning ability and memory (Fig. 3).

Discussion

Increasing evidence has demonstrated that the GABAergic system is involved in the pathogenesis of anxiety. Drugs and GABA analogs can significantly reduce the anxiety-like effects through affecting the neurotransmitter metabolism (22-24). The open-field and EZM tests were used in the current study to evaluate the anxiety-like behaviors of GAT-1$^{-/-}$ mice. When compared with the WT mice, the experimental results indicated that the GAT-1$^{-/-}$ mice showed decreased anxiety-like behaviors ($P<0.05$). The results obtained are consistent with a previous study which demonstrated that in tests for anxiety-like behaviors, such as the light-dark exploration test, emergence test or elevated-plus maze, the GAT-1$^{-/-}$ mice were prone to exhibit reduced anxiety (25). A GAT-1 deletion is hypothesized to cause an enhanced concentration of intracephalic GABA, which results in hyperactivity of GABAergic neurons and a consequent reduction in anxiety-like behaviors. A mutant of GAT-1 (SCL6A1) has been previously reported to be involved in the pathogenesis of anxiety (26).

A definite association between the GABAergic system and cognitive function has been established (12,13); however, the mechanism remains unclear. Clinical evidence suggests that tiagabine, a GABA reuptake inhibitor, can improve verbal memory when used as an adjunctive therapy in the treatment of convulsions (27). Similarly, NNC-711 (an analog of tiagabine) can also enhance cognitive function (28); however, there is contradictory evidence showing that tiagabine impaired the spatial learning of rats in the Morris water maze (29). At present, whether GAT-1 inhibitors are able to enhance or impair cognitive function remains controversial. In the present study, GAT-1 gene deletion was shown to result in impaired spatial learning and memory ability in GAT-1$^{-/-}$ mice. Cognitive behavioral tests, such as passive avoidance and contextual fear conditioning, have previously demonstrated that GAT-1$^{-/-}$ mice exhibit impaired hippocampus-dependent learning and memory (12). In addition, the cognitive function of GAT-1 overexpressing transgenic mice was found to be impaired in conditioned avoidance and novel object recognition tasks (30). These results indicated that GAT-1 can antiport and release GABA in normal and pathological conditions (31,32); however, the specific mechanism of GAT-1 antiport is not clear. Further research may help to clarify the function of GAT-1 in the modulation of excitatory and inhibitory amino acids in the brain.

Synaptic plasticity is considered to be one of the cellular mechanisms of learning and memory, and long-term potentiation (LTP) is an important form of synaptic plasticity (33). GAT-1 disruption has been demonstrated to specifically impair theta-burst stimulation-induced LTP and hippocampus-dependent learning and memory (15). Recent advances have revealed that GAT-1 heterozygous mice (GAT-1$^{+/-}$) manifested enhanced learning and memory ability through two behavioral experiments, namely the passive avoidance paradigm and the Morris water maze (34). By recording the field potential in the CA1 area of the hippocampus of three genetic phenotypes (GAT-1$^{+/+}$, GAT-1$^{+/-}$ and GAT-1$^{-/-}$), GAT-1$^{-/-}$ mice were found to have a decreased LTP in the hippocampus, while GAT-1$^{+/-}$ mice exhibited enhanced LTP. The results indicated that changing the activity of GAT-1 can alter the LTP in the CA1 region of the hippocampus (34). Therefore, the differing extent of GAT-1 deficiency may result in distinct effects on GABA metabolism. GAT-1$^{+/-}$ mice exhibited increased learning and memory, while homozygous GAT-1$^{-/-}$ mice exhibited impaired hippocampus-dependent learning and memory (34). Only a moderate reduction in GAT-1 activity caused an enhancement of learning and memory in mice.

In conclusion, the present study demonstrated that GAT-1 was involved in anxiety-like behaviors and cognitive function; thus, the transporter may be a potential target for the treatment of anxiety in the future.

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