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Reduced Aggression in Mice Lacking GABA Transporter Subtype 1

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Dysregulation of the brain GABAergic system has been implicated in the pathophysiology of violence and aggression. As a key regulator of central GABAergic activity, dysfunction of the GABA transporter subtype 1 (GAT1) represents a potential mechanism mediating pathologic aggression. We provide evidence that GAT1−/− mice and GAT1+/− mice exhibit lower aggressive behavior both in home cage resident–intruder test and neutral arena resident–intruder test, compared to wild-type mice (GAT1+/+). The pharmacologic effects of the GAT1 inhibitor, tiagabine and the $GABA_A$ receptor antagonist, bicuculline have been assessed in GAT1+/+ mice: tiagabine inhibits attacks but bicuculline induces attacks. Compared to GAT1+/− and +/+ mice, the GAT1−/− mice displayed a normal circadian pattern of home cage activity, but more activity overall. Meanwhile, reduced testosterone concentration was found in GAT1−/− mice compared to GAT1+/+ mice but not in GAT1+/+ mice treated with tiagabine, suggesting that testosterone is not directly involved in GAT1 mediated aggressive behavior regulation. These results showed that GAT1 is an important target involved in the regulation of aggressive behavior in mice, and long-term dysfunction of GAT1 may also result in the alteration of testosterone secretion.

Key words: GABA; GABA transporter; aggression; gene knock out mouse

Aggression is a complex social behavior of which the biologic mechanisms are largely unknown. Recent pharmacologic and genetic studies have markedly expanded the list of neurotransmitters, hormones, cytokines, enzymes, growth factors, and signaling molecules that influence behavior of aggression (Nelson and Chiavegatto, 2001). Among these, gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the central nervous system (CNS), modulates profoundly aggressive behavior in a range of species (Miczek et al., 2003). A number of studies have found a negative association between brain levels of GABA and aggressive-prone behavior (DaVanzo and Sydow, 1979; Krsiak et al., 1981; Poshivalov, 1981). Lower levels of GABA, particularly in the olfactory bulbs and striatum, were found in highly aggressive individual rats, mice, and hamsters (Earley and Leonard, 1977; Potegal et al., 1982; Haug et al., 1984). Muscimol and baclofen, GABA agonists at the $GABA_A$ and $GABA_B$ receptors respectively, are effective inhibitors of aggressive behavior (Delini-Stula and Vassout, 1978; Cheu and Siegel, 1998) indicating both the $GABA_A$ and the $GABA_B$ receptors are involved in the aggression suppressing effects of GABA.

The duration and intensity of GABAergic transmission are mainly controlled by the GABA reuptake activity of GABA transporters (GATs), located in the plasma membrane of neuronal cells and glial cells. There are four subtypes of GABA transporters identified presently (GAT1–4). GAT1 is the major subtype present at both synaptic and extrasynaptic sites in the brain (Guastella et al., 1990; Radian et al., 1990; Borden, 1996). Tiagabine, a specific GAT1 inhibitor used clinically for epilepsy treatment, was reported to reduce the symptoms of rage and aggression in a primary clinical trial (Kaufman et al., 2002; Hoffman, 2005). However, an opposing effect of tiagabine on aggressive behavior was also reported in an

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earlier clinical trial (Sveinbjornsdottir et al., 1994). These findings hint that GAT1 is another important modulator in mediating aggressive behavior. To elucidate the role of GAT1 in aggressive behavior, GAT1 knockout mice (GAT1−/−) were used to study the effect of this mutation on aggressive behavior. The methodologic approach used most frequently in the laboratory to induce mice to fight is to isolate males for some time, ranging from 24 hr to 8 weeks and subsequently, to confront the isolate males with a group-housed stranger in an unfamiliar test arena (neutral arena) or in the isolated home cage (Malick, 1979; Miczek et al., 2001). We show that the GAT1−/− mice exhibit less aggressive behavior in both home cage resident–intruder test and neutral arena resident–intruder test when they were compared to that of wild-type mice. It is documented that castrated mice rarely display aggressive behavior, and administration of testosterone can restore their aggression (Gandelman, 1980). Because the causal link between testosterone and aggression exists (Nelson and Chiavegatto, 2001), the plasma testosterone level in GAT1 knock out mouse was also measured and found to be lower. These results indicate that GAT1 plays an important role in regulation of aggressive behavior, and suggest that GAT1−/− mice represent a useful genetic animal model to understand the relationship between aggressive behavior and the function of GABA system.

MATERIALS AND METHODS

Subjects

GABA transporters subtype 1 knockout mice were generated as described previously (Cai et al., 2006). GAT1 knockout heterozygotes (GAT1+/−) from chimeric mice were crossed with C57BL/6J mice for another two generations. Then heterozygotes were intercrossed to give homozygous, heterozygous, and wild-type mice for further study. Mice were maintained under specific-pathogen-free conditions until up to 12–18 weeks of age before undergoing experiments. Male GAT1 knockout homozygous (GAT1−/−), heterozygous (GAT1+/−) and wild-type control (GAT1+/+) mice were individually housed in a temperature controlled and sound-proof vivarium under a 12-hr light/dark cycle (lights on at 7:00 AM). The age and body weight were matched in the behavioral test. All behavioral experiments were conducted in an isolated testing room to avoid environmental disturbance. The animals were placed in the test room at least 1 week before testing, allowing them to acclimate to the experimental environment. Naive mice were used in each behavioral experiment. All animal experiments described in this study were approved by the Institutional Animal Care and Use Committee.

Aggression Test

Aggression against intruder in home cage. Experimental male mice were housed individually for 6–8 weeks. In the test, a C57BL/6J adult male mouse (intruder) was placed into the home cage of the experimental male mouse (GAT1+/+, GAT1+/− or GAT1−/−) ($n = 8$ per group). Home cages were standard polycarbonate cages (28 cm × 17 cm × 12 cm), which were not cleaned for at least 1 week before testing. The latency to first attack, the total number of bite attacks, and the duration of social interest behaviors carried out by the resident mouse were recorded by an experienced observer who remained blind to the animals' genotype. Sniffing, nosing, grooming, and crawling over each other are considered social interest behavior. The aggression tests lasted for 15 min. A second encounter was carried out 1 week after the first. The latency to first attack was recorded as 15 min if no attack of the intruder occurred during the test session.

To test the effect of drugs on the aggressive behavior of mice, the wild-type male mice were treated intraperitoneally with tiagabine (4 mg/kg) or bicuculline (10 mg/kg) using a volume of 10 ml/kg. Bicuculline was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in saline. Tiagabine was a kind gift from Shanghai Celstar Research Center for Biotechnology (Shanghai, China) and also dissolved in saline. Forty-five minutes later, an intruder was placed into the home cage of the experimental male mouse and aggressive behavior was assessed as described above.

Aggression in neutral arena. Experimental male mice were individually housed 6–8 weeks. In the test, a C57BL/6J adult male mouse and an experimental male mouse were simultaneously introduced into a clear polycarbonate cage (28 cm × 17 cm × 12 cm) ($n = 7 \sim 8$/group). The floors of the cages were covered with 2–3 cm of pine shavings. The aggressive behaviors of the experimental mouse were recorded as described above.

Home Cage Activity

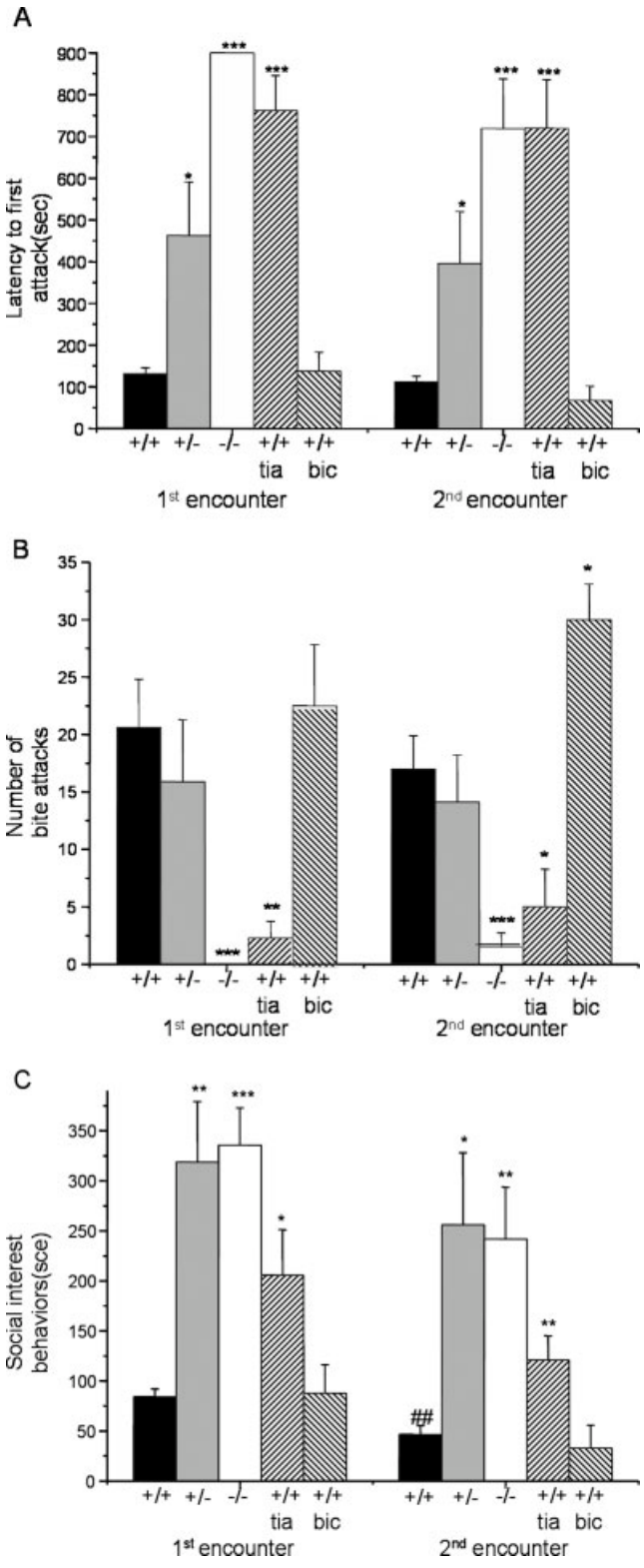
Home cage activity was assessed in mice that were individually housed in standard mouse cages (28 cm × 17 cm × 12 cm) and provided with nesting material. After a 24-hr acclimation period, activity was monitored and recorded by a video camera fixed above the cage for 5 min every 2 hr in a day ($n = 8$/group). Videotape was analyzed with a video-tracking system (Morris Maze Analyzer, V 1.1 by BGB).

Testosterone Concentration

Blood samples were obtained from the retro-orbital sinus, and spun through conical tubes at 2,500 × g for 15 min at 4°C ($n^{+/+} = 6$, $n^{+/-} = 6$, $n-/- = 6$, $n^{+/+\ \text{tiagabine}} = 5$, 100 ± 7 days old, male). Blood plasma was separated and stored at −80°C until assayed. Testosterone measurements were carried out on serum using a testosterone immunoassay (R&D Systems) according to manufacturer's instructions.

Statistical Analysis

Genotype effects or drug treatment effects in WT mice were analyzed using one-way ANOVA with Tukey post-tests. Two-way ANOVA with Bonferroni post-tests were used to analyze within–subject resident–intruder scores over two encounters and home cage activity over time; $P < 0.05$ set the threshold for a statistically significant difference. Wild-type, heterozygous, and homozygous GAT1-deficient mice are designated as +/+, +/−, and −/−, respectively.



RESULTS

Aggression Tests

Aggression against intruder in home cage. Aggressive behavior can be evaluated in male mice through an intruder-aggression test in the home cage, in which they defend their territory against an intruding unfamiliar male. Reduced aggressive behavior toward the unfamiliar intruder was observed in both GAT1+/− and GAT1−/− resident mice. As shown in Figure 1A, during the first encounter, the latency to attack the intruder of GAT1−/− mice ($n = 8$) is longer than 15 min ($F[1,14] = 2{,}831$; $P < 0.001$; −/− vs. +/+). GAT1+/− mice also showed a significant longer latency to attack the intruder ($F[1,14] = 6.6$; $P < 0.05$; +/− vs. +/+) than that of wild-type controls. During the second encounter, a similar result is shown, but GAT1−/− mice showed a shorter latency to attack the intruder because some made attacks. In Figure 1B, GAT1−/− mice did not even attack by biting ($F[1,14] = 24.2$; $P < 0.001$; −/− vs. +/+) and GAT1+/− mice also made fewer bite attacks (although it did not reach significance) than that of wild-type controls in the first encounter. During the second encounter, some of the GAT1−/− mice made some attacks but the aggressive behavior level was still significantly lower than that of GAT1+/+ ($P < 0.001$, two-way ANOVA, genotype × encounter). However, GAT1+/+ and +/− mice showed no significant change in the total number of attacks across the two encounters (Fig. 1B). Time spent on sniffing, grooming, and crawling over the intruder was significantly longer in GAT1−/− and +/− mice than that of GAT1+/+ on both the first ($F[1,14] = 43.9$; $P < 0.001$; −/− vs. +/+; $F[1,14] = 14.7$; $P < 0.01$, +/− vs. +/+) and second ($F[1,14] = 13.7$, $P < 0.01$, −/− vs. +/+; $F[1,14] = 8.4$;

Fig. 1. GAT1−/− mice show reduced aggression in resident–intruder test in home cage ($n = 8$/group). **A:** GAT1−/− mice were slower to first attack the intruder than GAT1+/+ controls on both the first and second encounters. The wild-type mice treated with tiagabine showed significantly slower to the first attack, but not mice treated bicuculline. The latency to first attack is depicted as 15 min if the attack of the intruder does not happen in the overall test process. Data are expressed as mean (±SEM) latency to first attack initiated by experimental animals. **B:** GAT1+/+ and +/− made significantly more bite attacks than GAT1−/− mice on both the first and second encounters. The wild-type mice treated with tiagabine showed significantly less bite attacks, and mice treated bicuculline made more attacks on the second encounter but not on the first encounter, as compared to GAT1+/+ controls. Data are expressed as mean (±SEM) total number attacks initiated by experimental animals. **C:** GAT1−/− and +/− mice have a significantly higher social behavior level both on the first and second encounters, compared to GAT1+/+ mice. The wild-type mice treated with tiagabine showed significantly higher social behavior level, but not mice treated bicuculline. All the three genotype of mice had a slight social behavior reduction on the second encounter. Data are expressed as mean (±SEM) total time of social interest behaviors with the experimental animals. ⋆$P < 0.05$; ⋆⋆$P < 0.01$; ⋆⋆⋆$P < 0.001$ vs. GAT1+/+ mice on the same encounter (one-way ANOVA), ##$P < 0.01$ vs. the same genotype on the first encounter (one-way ANOVA). tia, tiagabine; bic, bicuculline.

$P < 0.02$; +/− vs. +/+) encounter, but there was no significant difference between GAT1+/− and GAT1+/+ (Fig. 1C). As expected, mice treated with tiagabine were showed slower to attack the intruder (Fig. 1A), made fewer bite attacks (Fig. 1B), and spent more time in social behaviors (Fig. 1C) on both the first and second encounter. Mice treated with bicuculline were faster (although it did not reach significance) to attack and made more bite attacks on the second encounter ($F[1,12] = 9.3$; $P < 0.02$) but not the first encounter, as compared to GAT1+/+ controls.

Aggression in neutral arena. This paradigm was designed to test offensive aggression in a novel, neutral environment. When paired with a stimulus animal, GAT1−/− resident mice showed reduced aggressive behavior, consistent with the home cage intruder test. As shown in Figure 2A,B, GAT1−/− mice were significantly slower to attack the stimulus animals ($F[1,13] = 4.9$; $P < 0.05$) and made significantly fewer bite attacks ($F[1,13] = 16.7$; $P < 0.01$) than GAT1+/+ mice during the first encounter. GAT1+/− mice were also slower to attack the intruder ($F[1,13] = 3.1$; $P = 0.1$) and made fewer bite attacks ($F[1,13] = 4.6$; $P = 0.053$) than GAT1+/+ controls. During the second encounter, both GAT1−/− and +/− mice were again significantly slower to attack the stimulus mice and made significantly fewer total attacks, as compared to +/+ controls. Aggressive behavior increased in GAT1+/+ mice over the two encounters, with a significant reduction in the latency to attack and a higher total number of attacks (no significance) on the second encounter. In contrast, GAT1−/− mice showed no obvious change in the latency to attack but made fewer bite attacks on the second encounter than the first. As shown in Figure 2C, there was no significant difference in social interest behaviors on the first encounter between genotypes, but GAT1−/− mice show a significant higher social interest behaviors level on second encounter ($F[1,12] = 15.1$; $P < 0.01$; −/− vs. +/+), as compared to +/+ mice.

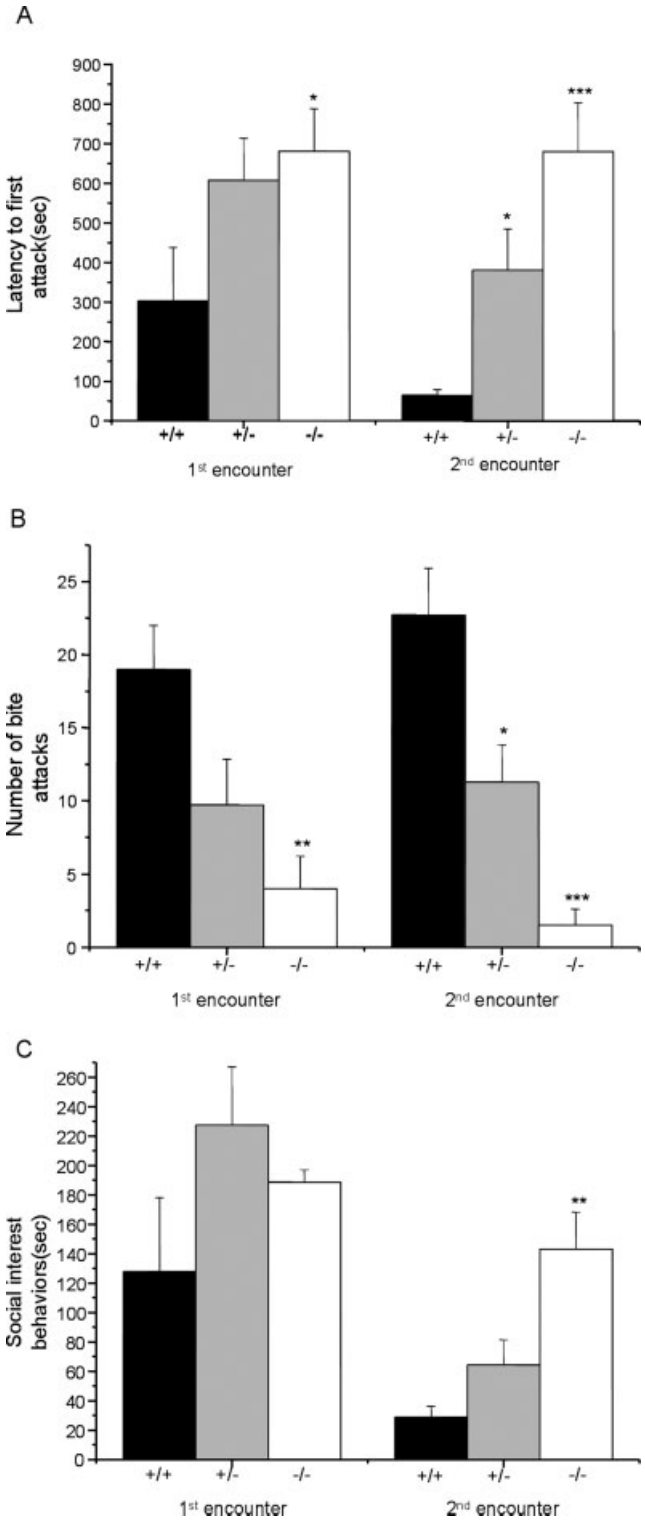


Fig. 2. GAT1−/− mice show reduced aggression in neutral arena ($n = 7 \sim 8$/group). **A:** GAT1−/− mice were slower to first attack the intruder than GAT1+/+ littermate controls on both the first and second encounters. GAT1+/− and +/+ mice, but not GAT1−/− mice, were quicker to first attack on the second encounter, as compared to the first. The latency to first attack is depicted as 15 min if the attack of the intruder does not happen in the overall test process. Data are expressed as mean (±SEM) latency to first attack initiated by experimental animals. **B:** GAT1−/− and GAT1+/− mice made fewer bite attacks than GAT1+/+ controls on both the first and second encounters. Data are expressed as mean (±SEM) total number attacks initiated by experimental animals. **C:** There were no genotype differences in social interest behaviors on the first encounter, but there is a significant effect of test experience for the second encounter. GAT1+/− and +/+ mice, but not GAT1−/− mice, have a notable reduction in social interest behaviors on the second encounter. GAT1−/− mice show a slight decrease, but a significant higher social interest behaviors level on the second encounter, compared to GAT1+/− and +/+ mice. Data are expressed as mean (±SEM) total time of social interest behaviors with the experimental animals. ★$P < 0.05$; ★★$P < 0.01$; ★★★$P < 0.001$ vs. GAT1+/+ mice on the same encounter (one-way ANOVA).

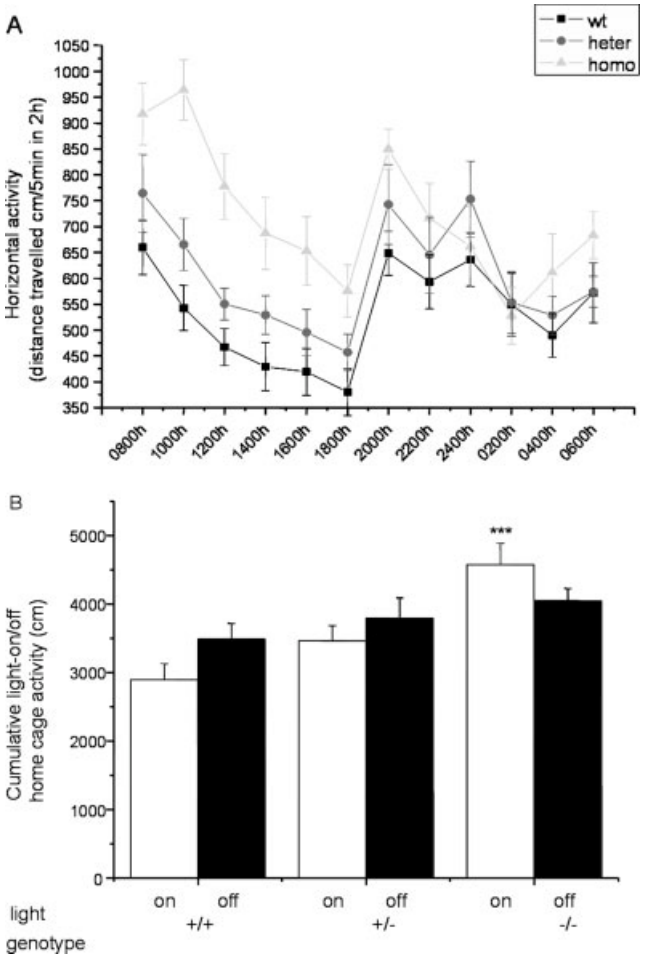


Fig. 3. GAT1−/− mice show increased home cage activity ($n = 8$/group). **A:** GAT1−/− mice show higher level of home cage activity, as compared to GAT1+/+ littermate controls ($P < 0.001$, −/− vs. +/+; $P < 0.01$, +/− vs. +/+; two-way ANOVA followed by Bonferroni post-tests [genotype × time]). **B:** Cumulative home cage activity was higher in GAT1−/− as compare to GAT1+/+ controls ($P < 0.001$, −/− vs. +/+. two-way ANOVA followed by Bonferroni post-tests [genotype × light on/off]).

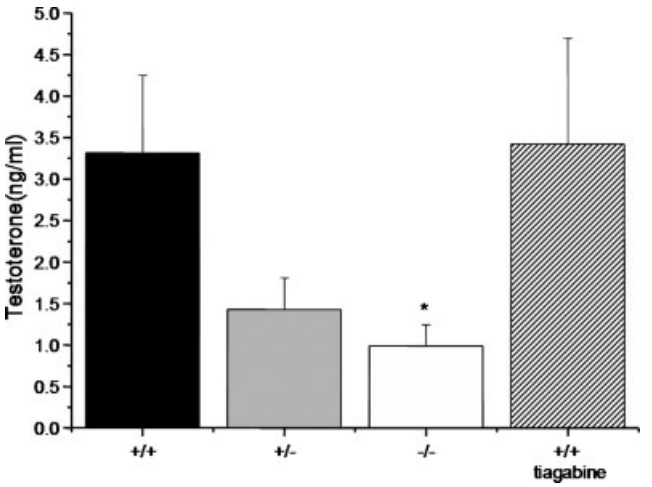


Fig. 4. GAT1−/− mice show reduced blood testosterone concentration. ($n^{+/+} = 6$, $n^{+/-} = 6$, $n^{-/-} = 6$, $n^{+/+\ \text{tiagabine}} = 5$). Testosterone concentrations assay shows the remarkable difference between GAT1+/+ and GAT1−/− mice, but not between GAT1+/+ and GAT1+/+ mice treated with tiagabine. *$P < 0.05$ vs. GAT1+/+ mice (one-way ANOVA).

Home Cage Activity

Figure 3A shows the horizontal activity in different time periods over the course of a day. Distance travelled was measured for 5 min every 2 hr. The fluctuation trends of curves for three genotypes are similar. There are mainly two activity peaks during the 24-hr test for all the genotypes. One is between 8:00–10:00 AM and the other is at 8:00 PM. Both of them are about 2 hr after the lights-on/-off switch. For GAT1−/− mice, horizontal activity reaches first peak at 10:00, traveling 964 cm/5 min, which is significantly higher when compared to GAT1+/+ controls. During daytime from 10:00 AM to 6:00 PM, horizontal activity subsides gradually, reaching the bottom at 6:00 PM. The second peak appears at 8:00 PM with 849 cm/5 min, which remains notably higher when compared to that of GAT1+/+ and +/− controls. As shown in Figure 3A,B, GAT1−/− mice show significant higher level of home cage activity during the lights-on period as compared to GAT1+/+ ($P < 0.001$, −/− vs. +/+; $P < 0.01$, +/− vs. +/+; two-way ANOVA followed by Bonferroni), whereas in the lights-off period there was no significant difference among three genotypes. For GAT1+/−, the curve is between that of GAT1−/− and +/+ with a trend corresponding to GAT1+/+ controls. The cumulative home cage activity confirms that overall activity level of GAT1−/− is significantly higher than that of GAT1+/+ and +/− controls, whereas there is no such notable difference between +/− and +/+ controls (Fig. 3B).

Testosterone Concentration

Strong evidence exists in C57BL/6J mice for a causal link between testosterone and aggression (Nelson and Chiavegatto, 2001). An enzyme-linked immunosorbent assay (ELISA) was carried out to measure the blood testosterone concentrations in wild-type and mutant mice. As shown in Figure 4, blood testosterone concentration of GAT1+/+ mice is 3.3 ± 0.94 ng/ml, whereas it is only 0.99 ± 0.25 ng/ml in GAT1−/−mice ($F[1,10] = 5.7$; $P < 0.05$; −/− vs. +/+).

DISCUSSION

The present findings show that male GABA transporter subtype 1 (GAT1) knockout mice showed reduced aggression in two aggression tests: home cage resident–intruder and neutral arena resident–intruder. GAT1−/− mice showed a longer latency to attack an intruder, and attacked the intruder with less frequency than +/+ controls. GAT1+/− mice exhibited an intermediate profile

in these aggression tests: an intermediate attacking frequency and an intermediate latency to make the first attack. These findings suggest that deletion of the GAT1 reduces isolation-induced aggression in a gene dose-dependent manner.

Carrying out multiple determinations of aggressive behavior until a stable level has been reached (Miczek et al., 2001) is recommended. Mice were tested on a second aggression test encounter. GAT1+/+, +/−, and −/− mice did not show significant increases in aggression with repeated testing, although some of them had a non-significant trend for a higher number of bite attacks on the second encounter relative to the first. GAT1+/− mice again exhibited an intermediate profile on the second encounter. Moreover, GAT1−/− mice showed higher levels of non-aggressive, social behaviors in these tests, further providing convincing evidence of reduced aggressive behavior in GAT1−/− mice.

To mimic the aggressive phenotype of GAT1−/− mice, tiagabine, the GAT1 inhibitor, was used. We found that tiagabine inhibits attack number in the resident–intruder test in home cage, which is consistent with the phenotype of GAT1−/− mice. GABAergic neurotransmission has been implicated in control of emotional behavior including fear, anxiety, and aggression (Siegel et al., 1999). Adams et al. (1993) and Roeling et al. (1993) gave evidence for involvement of GABA receptors in hypothalamic aggression in rats. Drugs that activate $GABA_A$ receptors, such as muscimol, have anti-aggressive effects in animal models (Delini-Stula and Vassout, 1978). Suppressive effects on defensive rage were also noted for chlorpromazine and pentobarbital (Baxter, 1968; Maeda, 1976). This may explain the effect of the tiagabine on mice aggressive behavior, because the GABA concentration in synaptic cleft will be raised after GAT1 being blocked. It was reported GAT1 deficiency would also lead to enhanced extracellular GABA levels and results in an over-activation of $GABA_A$ receptors (Jensen et al. 2003). These findings provide strong evidence that reduced aggression in male GAT1−/− mice is mediated through over-activation of $GABA_A$ receptors.

Stork et al. (2000) reported recently that GAD65−/− mice showed reduced aggression. The 65-kDa isoform of glutamic acid decarboxylase (GAD65) is believed to play an essential role for GABA synthesis in the central nervous system. However, in the juvenile GAD65−/− mice, GABA content seems to be normal in the striatum, hippocampus, cerebellum, and cerebral cortex. They show a pronounced reduction of cofactor-induced GAD activity and an increased susceptibility to pentylenetetrazol- and picrotoxin-, as well as stress-induced seizure (Asada et al., 1996). Therefore, the reduced aggression in GAD65−/− mice may in part result from the development of seizure activity and accompanying brain damage. Reduced aggressive behavior of GAT1−/− mice may be due to over-activation of GABAergic inhibition. Further and more detailed studies will be necessary to clarify why aggressive behavior is similar in GAD65−/− and GAT1−/− mice.

Differences in locomotor activity can contribute to apparent differences in aggressive behavior. GAD65−/− mice showed reduced aggressive behaviors and increased locomotor activity compared to GAD65+/+ and +/− mice (Stork et al., 2000). Neutral endopeptidase (NEP) −/− mice showed increased aggressive behaviors and decreased locomotor activity (Fischer et al., 2000). In a similar way, the GAT1−/− mice showed significantly higher levels of home cage activity as compared to GAT1+/− and +/+ controls. However, the relationship between locomotor activity and aggression in rodents remains unclear, and there is an opposing report that the reduced locomotor activity is associated with low levels of aggression in 5-HTT−/− mice (Holmes et al., 2002).

In vertebrate species, males are usually more aggressive than females (Giammanco et al., 2005). It is well-documented that castration eliminates the aggressive behavior in males, and the neuroendocrine mediators of aggressive behavior are primarily androgens. In nonhuman animals, strong evidence for a causal link between testosterone and aggression exists (Nelson and Chiavegatto, 2001). For this reason we measured the level of testosterone in GAT1+/+ and −/− mice and low level of testosterone was measured in GAT1−/− mice. However, GAT1+/+ mice treated with tiagabine did not show statistically significant change in testosterone level, as compared to control mice. Such difference may be attributed to the different time of GAT1 absence; the lifelong absence of GAT1 in GAT1−/− mice (or the long-term situation of reduced aggressive behavior in GAT1−/− mice) may reduce the testosterone's secretion, but not in the acute tiagabine treatment. These results do not support the idea that the reduced aggressive behavior is caused primarily by the lower level of testosterone observed in GAT1−/− mice. We believe that over-activation of GABAergic inhibition can directly cause the reduced aggressive behavior observed in GAT1−/− mice, however, the low level of testosterone caused by GAT1 deficiency may also contribute partly to the phenotype of lower aggression in mutant mice.

In conclusion, we provide the evidence that GAT1 gene function is involved in the regulation of aggressive behavior. The reduced testosterone concentration is also observed in GAT1−/− mice. GAT1−/− mice provide a useful animal model for studying the role of GAT1 in aggressive behavior and, more generally, how perturbation of the GABAergic system homeostasis impacts aggressive behaviors.

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