

# Decreased locomotor activity in mice expressing tTA under control of the CaMKII $\alpha$ promoter

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**Transgenic mice in which the tetracycline transactivator (tTA) is driven by the forebrain-specific calcium-calmodulin-dependent kinase II $\alpha$  promoter (CaMKII $\alpha$ -tTA mice) are used to study the molecular genetics of many behaviors. These mice can be crossed with other transgenic mice carrying a transgene of interest coupled to the tetracycline-responsive promoter element to produce mice with forebrain-specific expression of the transgene under investigation. The value of using CaMKII $\alpha$ -tTA mice to study behavior, however, is dependent on the CaMKII $\alpha$ -tTA mice themselves lacking a behavioral phenotype with respect to the behaviors being studied. Here we present data that suggest CaMKII $\alpha$ -tTA mice have a behavioral phenotype distinct from that of their wild-type (WT) littermates. Most strikingly, we find that CaMKII $\alpha$ -tTA mice, both those with a C57BL/6NTac genetic background (B6-tTA) and those with a 129S6B6F1/Tac hybrid genetic background (F1-tTA), exhibit decreased locomotor activity compared with WT littermates that could be misinterpreted as altered anxiety-like behavior. Despite this impairment, neither B6-tTA nor F1-tTA mice perform differently than their WT littermates in two commonly used learning and memory paradigms – Pavlovian fear conditioning and Morris water maze. Additionally, we find data regarding motor coordination and balance to be mixed: B6-tTA mice, but not F1-tTA mice, exhibit impaired performance on the accelerating rotarod and both perform as well as their WT littermates on the balance beam.**

Keywords: Anxiety, calcium-calmodulin-dependent kinase II $\alpha$  (CaMKII $\alpha$ ), learning and memory, locomotor activity, tetracycline transactivator (tTA).

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Since its development by Bujard and colleagues (Furth *et al.* 1994; Gossen & Bujard 1992), the tetracycline-controlled gene expression system has served as an invaluable tool for the study of gene functions. The system has two components. The first component is the tetracycline transactivator (tTA). The tTA is the expression product of the fusion gene formed from the union of the repressor (*tetR*) of the *Escherichia coli* Tn10 tetracycline-resistance operon and a DNA sequence encoding a carboxy-terminal portion of protein 16 of herpes simplex virus (VP16), a strong transcription activator. The second component is the tetracycline response element (TRE) coupled to the minimal human cytomegalovirus promoter and a target gene. The TRE consists of multiple copies of tetracycline operator (*tetO*) sequences. When the two components are brought together, tTA binds the TRE and activates transcription of the target gene. In the so-called 'Tet-off' system, the presence of tetracycline (or its analogue doxycycline) prevents binding of tTA to the TRE and thus prevents the expression of the target gene. Thus temporal restriction of target gene expression can be achieved by adding tetracycline or doxycycline to the system and target gene expression can be made cell-specific by driving tTA expression with a promoter that is only active in a particular type of cell (Mansuy & Bujard 2000).

The tetracycline-controlled gene expression system was originally developed as a tool to study gene function in mammalian cell culture (Gossen & Bujard 1992). Subsequently, it was implemented to generate transgenic mice that allow for spatial and temporal control of target gene expression (Furth *et al.* 1994). Mayford *et al.* (1996) were the first to successfully apply this technology to study gene function in the brain and behavior. They developed mice that used the calcium-calmodulin-dependent kinase II $\alpha$  (CaMKII $\alpha$ ) promoter, a forebrain-specific promoter, to drive expression of tTA. These mice, the CaMKII $\alpha$ -tTA mice, have become a popular and powerful tool for the study of the molecular genetics of learning and memory (Bejar *et al.* 2002; Isiegas *et al.* 2006; Mayford *et al.* 1996; Ramsden *et al.* 2005) and other behaviors (Chen *et al.* 2007; Gross *et al.* 2002; Yamamoto *et al.* 2000). Inherent in the experimental design for studies that use the CaMKII $\alpha$ -tTA mice to investigate gene-behavior interactions is the assumption that the CaMKII $\alpha$ -tTA mice lack an overt behavioral phenotype. Here we present a series of

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experiments showing that the CaMKII $\alpha$ -tTA mice themselves exhibit a behavioral phenotype distinct from that of their wild-type (WT) littermates that may confound certain behavioral experiments.

The data presented here represents the results of two studies designed and executed by two different, independent groups at the University of Michigan and analyzed together *post hoc*. Despite the fact that the two studies used mice with different genetic backgrounds and used different procedures and housing conditions, the results of both studies are remarkably consistent. Together, the studies show that CaMKII $\alpha$ -tTA mice exhibit less locomotor activity than their WT littermates.

## Materials and methods

### Mice

Open-field, light–dark box and elevated plus maze data were obtained from mice maintained on a C57BL/6NTac background by successively crossing offspring carrying the CaMKII $\alpha$ -tTA transgene (Mayford *et al.* 1996) with C57BL/6NTac WT mice purchased from Taconic Farms (Hudson, NY, USA) for more than 10 generations. Testing in this strain was performed on mice carrying the CaMKII $\alpha$ -tTA transgene and their WT littermates (both male and female) between 8 and 16 weeks of age. Mice were housed under uniform conditions including a 14 h–10 h light–dark cycle with lights on at 0600 h, average temperature of 22°C, and *ad libitum* food and water. Additionally, these mice were moved from group to single housing 7 days prior to testing. This was performed to be consistent with other on-going experiments in the laboratory, which generally require the collection of blood for assay of plasma adrenocorticotropic hormone (ACTH) levels at the conclusion of the experiment, which – in group housed animals – is subject to an order effect such that the last mouse taken from the cage always has higher levels of ACTH compared with the first (Burrows *et al.* 1998). Although the impact of isolation upon measures of anxiety in mice is somewhat controversial with evidence suggesting that isolation has no effect (Manzaneque *et al.* 2002; Rodgers & Cole 1993) or a partial effect (Voikar *et al.* 2005) or a significant effect (Guo *et al.* 2004), single-housed animals do not exhibit significant increases in stress levels as measure by corticosterone levels (Hunt & Hambly 2006). Testing with these mice occurred between 0700 h and 1200 h. Open-field testing was carried out on days 1–3, elevated plus maze on day 5 and light–dark box on day 8. Males and females were tested separately on different days by observers blind to genotype; male and female data were analyzed separately.

Rotarod, Morris water maze (MWM), Pavlovian fear conditioning and balance beam data from C57BL/6NTac mice were obtained from mice with the same genetic background and of similar age as those described above. The mice used in these paradigms, however, differed in that they were housed under a 12 h–12 h light–dark cycle with lights on at 0600 h, were housed in groups of 3–5 with same-sex siblings and were tested between 1300 h and 1700 h. To minimize potential confounds introduced by test order (McIlwain *et al.* 2001), experiments were performed in the following order with 2–4 days rest between each experiment: MWM, Pavlovian fear conditioning in one group and rotarod and balance beam were performed in a separate group of mice. Although both male and female mice were also used in these paradigms, they were tested at the same time and their data analyzed together.

All data from 129S6B6F1/Tac hybrid mice were obtained from mice generated by crossing male mice carrying the CaMKII $\alpha$ -tTA transgene on a C57BL/6NTac background with female 129S6/SvEvTac (formerly 129/SvEvTac) WT mice purchased from Taconic Farms. Mice were housed under uniform conditions including a 12 h–12 h light–dark cycle with lights on at 0600 h, average temperature of 22°C and *ad libitum* food and water. Mice were housed together in groups of 3–5 with same-sex siblings. Experiments were carried out on 8 to 16-week-old mice carrying the CaMKII $\alpha$ -tTA transgene and their WT

littermates, approximately equal numbers of male and female mice were used in all experiments. To minimize potential confounds introduced by test order (McIlwain *et al.* 2001), experiments were performed in the following order with 2–4 days rest between each experiment: rotarod, open field, light–dark box, elevated zero maze, MWM and Pavlovian fear conditioning. In addition, a separate group of 129S6B6F1/Tac hybrid mice were tested the rotarod and subsequently tested on the balance beam 4 days later. Rotarod data from this group were pooled with the mice from the first group for analysis. All experiments were conducted according to National Institutes of Health guidelines for animal care and were approved by the University Committee on Use and Care of Animals of the University of Michigan.

### Open field

#### C57BL/6NTac mice

The open-field experiments were similar to those previously described (Rozeboom *et al.* 2007). In brief, an automated Plexiglas Digiscan Activity Monitor chamber (41.5 × 41.5 × 30 cm; AccuScan Instruments, Columbus, OH, USA) in room lit by indirect white light was used for this test. Mice were placed singly into the middle of the chamber and allowed to explore for 30 min. Total distance traveled was measured by infrared beam breaks.

#### 129S6B6F1/Tac mice

The open-field experiments were conducted as previously described (Hebda-Bauer *et al.* 2004; McKinney & Murphy 2006). The chamber consisted of a white acrylic box (71 × 71 × 30 cm) in room lit by indirect white light. Mice were placed singly in the center of the chamber and allowed to explore for 5 min. Total distance traveled was measured using the video signals from digital cameras sent to a desktop PC and processed online using ACTIMETRICS LIMELIGHT software (Actimetrics, Wilmette, IL, USA).

### Light–dark box

#### C57BL/6NTac mice

The light–dark box experiments were similar to those previously described (Hebda-Bauer *et al.* 2004). The light–dark box is 46 cm long with two-thirds of the length comprising the light compartment (made of white acrylic) and one-third comprising the dark compartment (made of black acrylic with a lid). Mice were placed in the dark compartment under indirect white lighting and their behavior was observed for 5 min. The number of light–dark transitions between the two compartments and per cent time in light side were scored by hand.

#### 129S6B6F1/Tac mice

The light–dark box used to test 129S6B6F1/Tac mice was the same as that used for C57BL/6NTac mice. Unlike the C57BL/6NTac mice, the 129S6B6F1/Tac mice were placed in the light compartment at the start of testing and observed for 10 min. The number of light–dark transitions between the two compartments and per cent time in light side were scored by using ACTIMETRICS LIMELIGHT software (Actimetrics).

### Elevated plus maze

#### C57BL/6NTac mice

The elevated plus maze experiments were performed essentially as described (Burrows *et al.* 1998; Wei *et al.* 2004). Briefly, the maze consisted of four arms (30 × 6 cm) arranged in a plus shape and elevated 50 cm from the floor. Two opposing arms are surrounded with 16 cm high clear Plexiglas walls (closed arms), whereas the open arms do not have walls. Mice were placed in the center of the maze facing an open arm at the start of testing and observed for 5 min. Latency to enter an open arm, time in the open arms and the per cent of total entries that were into an open arm were scored by hand.

### Elevated zero maze

#### 129S6B6F1/Tac mice

The elevated zero maze is composed of a 6-cm-wide ring with a 70 cm outer diameter and alternating walled and unwalled quadrants (San Diego Instruments, San Diego, CA, USA). The entire ring is elevated to a height of 70 cm. Mice were placed in the walled region at the start of the 5-min test and their movements tracked. Mice were considered to be in the open quadrant when all four paws were entirely on the open portion of the maze. Total distance traveled, percentage of the total distance traveled in an open quadrant and latency to first entry into an unwalled quadrant were scored using the video signals from digital cameras sent to a desktop PC and processed online using ACTIMETRICS LIMELIGHT software (Actimetrics).

### Rotarod

#### Both C57BL/6NTac and 129S6B6F1/Tac mice

The rotarod experiments were carried out in a similar fashion to that which has previously been described (McKinney & Murphy 2006). Mice were placed on the rotating drum of an accelerating rotarod (Ugo Basile/Stoelting accelerating rotarod, Chicago, IL, USA) and the time each mouse was able to walk on top of the drum was measured. The speed of the rotarod accelerated from 4 to 40 rpm over a 5-min period. Mice were given one trial per day for 5 days with a maximum time of 300 seconds (5 min). Latency to fall or first passive rotation was scored by hand.

### Balance beam

The balance beam consisted of an elevated (50 cm above table) beam leading from a brightly illuminated start area to an enclosed escape box (20 cm<sup>2</sup>). Two different-sized Plexiglas beams (round 11 mm diameter and square 5 mm width) were used. Beams were serrated for claw grip. Following four acclimation trials, four training trials (one trial per day) were administered. For each training trial, the number of hind foot slips were recorded (Carter *et al.* 1999).

### Morris water maze

The MWM was run as previously described (McKinney & Murphy 2006). The pool consisted of a 1.2 m diameter pool filled with water, which was made opaque with white non-toxic paint. Water was maintained at 25  $\pm$  2°C. The walls surrounding the pool were adorned with high-contrast posters for use as distal cues. For 10 days prior to training, mice were handled for 2–3 min once daily. During training, each training trial began with the mouse on the platform for 15 seconds. The mouse was then placed into the water facing the wall of the pool and allowed to search for the platform. The trial ended either when the mouse climbed onto the platform or when 60 seconds had elapsed. At the end of each trial, the mouse was allowed to rest on the platform for 15 seconds.

The C57BL/6NTac mice received six training trials a day for 5 days. Trials were administered in blocks of two with the second trial immediately following the first and the interblock interval equal to 1 h. The 129S6B6F1/Tac mice were given two trials per day (a single training block) for 6 days, with the starting position chosen pseudorandomly among six start positions.

The probe trial was conducted 24 h after the end of training on day 6 for C57BL/6NTac mice and day 7 for 129S6B6F1/Tac mice. During probe trials, the escape platform was removed and mice were placed in the pool at the start location directly opposite the platform and allowed to swim for 60 seconds. Mice were run in the visible platform version of the water maze 24 h following the probe trial on day 7 for C57BL/6NTac mice and day 8 for 129S6B6F1/Tac mice. The visible platform version consisted of a single day of training with six trials during which the platform was moved to new location after every two trials and marked with a distinct local cue (a flag). All MWM data were collected with digital cameras and sent to a desktop PC. ACTIMETRICS WATERMAZE software was used to process the collected data (Actimetrics).

### Pavlovian fear conditioning

Pavlovian fear conditioning was accomplished as previously described (McKinney & Murphy 2006). The Pavlovian fear conditioning apparatus (Med Associates Inc., St Albans, VT, USA) consisted of four conditioning chambers each with a stainless steel grid floor designed for mice, through which the unconditioned stimulus (US; foot shock) was delivered. The grid floor is over a stainless steel drop-pan, which was lightly cleaned with 95% ethyl alcohol to provide a background odor. The conditioning chambers were arranged in a 2  $\times$  2 configuration on a steel rack in an isolated room lit by adjustable indirect lighting, and each chamber was outfitted with an individual video camera. Fear was assessed by measuring freezing behavior. Freezing was defined as the absence of movement except that associated with respiration and was measured by subjecting the video signal to a sensitive global motion-detection algorithm (FREEZEFRAME and FREEZEVIEW software; Actimetrics). Freezing data are presented as per cent freezing, which is the amount of time an individual animal spent freezing divided by the duration of the trial and multiplied by 100.

C57BL/6NTac mice received three training trials (one trial per day) in which a 3 min baseline was followed by a 30 second tone, which co-terminated with a 2-second, 0.70 mA foot shock delivered via the grid floor. 129S6B6F1/Tac mice were similarly trained but received only two training trials (one trial per day) and a 0.50 mA foot shock. Mice were removed from the chambers 30 seconds following the shock. Twenty-four hours after the final training trial (on day 4 for C57BL/6NTac mice and day 3 for 129S6B6F1/Tac mice), contextual conditioning was assessed by returning mice to the same chambers and assessing freezing during a 5-minute trial in the absence of tone or shock. Cued conditioning was assessed on the following day (day 5 for C57BL/6NTac mice and day 4 for 129S6B6F1/Tac mice). For cued conditioning, the conditioning chambers were reconfigured by using white plastic inserts that covered the grid floor and walls to change the appearance and geometry of the chambers (i.e. semicircular instead of rectangular). In addition, the chamber was cleaned with 2% acetic acid (as opposed to 95% ethanol) to provide a novel background odor. After 2 minutes of baseline, freezing was measured in response to a 3-min tone.

### Statistical analysis

All data are presented as mean  $\pm$  SEM. Data from male and female C57BL/6NTac mice in the open field, light–dark box and elevated plus maze were analyzed separately by two-tailed unpaired *t*-tests between genotypes. In all experiments with 129S6B6F1/Tac mice and some of the experiments (accelerating rotarod, MWM and Pavlovian fear conditioning) with C57BL/6NTac mice, groups were not large enough for separate analysis of female and male subjects, so mice of the same genotype were pooled for analysis. Data from 129S6B6F1/Tac mice open field, light–dark box and elevated zero maze were analyzed by two-tailed *t*-tests between genotypes. Data from the accelerating rotarod, MWM and Pavlovian fear conditioning in both C57BL/6NTac and 129S6B6F1/Tac mice were analyzed by two-way repeated measures ANOVA with a between-subject factor for genotype and a repeated measure for training day. As data for these experiments were pooled and the numbers of male and female mice were not equal, it is possible that sex effects may have been mistaken for genotype effects; that is, genotype effects may have been underestimated or overlooked if they were sex dependent or if the baseline differences between female and male mice increased the variance in the data. Therefore, ANOVA models were used to check for the sex dependence of the genotype effects. A two-way ANOVA with between-subject factors for genotype and sex was used for open-field, light–dark box and elevated plus maze data and a three-way repeated measures ANOVA with between-subject factors for genotype and sex and a repeated measure for training day was applied to accelerating rotarod, MWM and Pavlovian fear conditioning data. These analyses showed only one measure in which there was a significant effect of sex – open to total distance ratio in the elevated zero maze – and this effect did not appear to account for the effect of genotype (Figure S3). Results were considered significantly different when  $P < 0.05$ . A small number of mice were eliminated from analysis in some paradigms because of data collection problems

(e.g. computer crash, mice falling from elevated maze) that resulted in a loss of data for that mouse on that trial; however, these mice were used for analysis in other paradigms in which their data were available.

**Results**

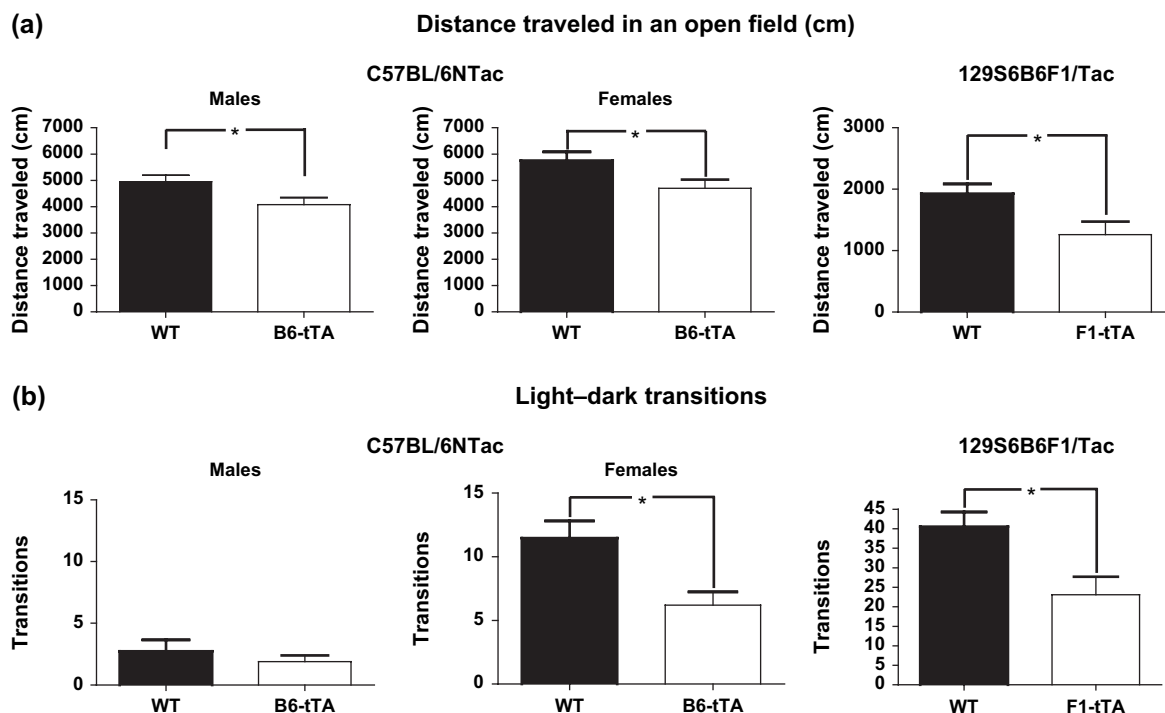
**CaMKII $\alpha$ -tTA mice exhibit decreased locomotion in the open field**

Figure 1a illustrates the data from the open-field test. CaMKII $\alpha$ -tTA mice with a C57BL/6NTac genetic background (B6-tTA mice) of both sexes exhibit a significant decrease in distance traveled during the 30-min open-field test compared with their WT littermates (Fig.1a). An unpaired *t*-test shows that B6-tTA males (*n* = 17) traveled a significantly shorter distance (4081.47  $\pm$  269.83 cm) in the open field than their male WT littermates (*n* = 19; 4947.16  $\pm$  245.09 cm; *t*<sub>1,35</sub> = 2.4, *P* < 0.05; Fig. 1a). Similarly, B6-tTA females (*n* = 19) traveled a significantly shorter distance (4399.79  $\pm$  1450.94 cm) in the open field than their female WT littermates (*n* = 20; 5752.55  $\pm$  1489.44 cm; *t*<sub>1,37</sub> = 2.87, *P* < 0.05; Fig. 1a). Despite being tested in an open field with different dimensions and for a different length of time, CaMKII $\alpha$ -tTA mice that had been crossed on to a 129S6B6F1/Tac genetic background (F1-tTA) exhibited a phenotype similar to B6-tTA in the open-field test. F1-tTA mice (*n* = 8) traveled significantly shorter distances than their

WT littermates (*n* = 12) in the 5-min open-field test. An unpaired *t*-test showed that the distance traveled by the F1-tTA mice was significantly less in the open field (1259.23  $\pm$  214.65 cm) when compared with their WT littermates (1927.07  $\pm$  156.18 cm; *t*<sub>1,18</sub> = 2.58, *P* < 0.05; Fig. 1a).

**CaMKII $\alpha$ -tTA mice exhibit decreased locomotion in the light-dark box**

Data from the light-dark box test are shown in Fig. 1b. As in the open field, CaMKII $\alpha$ -tTA mice exhibit decreased locomotion in the light-dark box. Female B6-tTA (*n* = 19), but not male B6-tTA mice (*n* = 20 compared with *n* = 19 WT littermates), transitioned fewer times between the compartments than their WT littermates (*n* = 20; B6-tTA = 6.23  $\pm$  4.50 transitions and female WT littermates = 12.55  $\pm$  7.48 transitions; *t*<sub>1,35</sub> = 3.19, *P* < 0.05 for females and B6-tTA = 1.9  $\pm$  0.49 transitions and WT littermates = 2.74  $\pm$  0.92 transitions; *t*<sub>1,37</sub> = 0.8, *P* > 0.05 for males; Fig. 1b). Neither male nor female B6-tTA mice differ from their WT littermates with respect to per cent time spent in the light side (B6-tTA = 2.40  $\pm$  1.17% and WT = 4.28  $\pm$  1.97%, *t*<sub>1,37</sub> = 0.8, *P* > 0.05 for males; B6-tTA = 16.05  $\pm$  4.70% and WT = 14.41  $\pm$  1.88%, *t*<sub>1,35</sub> = 0.33, *P* > 0.05 for females; data not shown). Although F1-tTA mice were started on the opposite side of the light-dark box from B6-tTA mice (light, rather than dark for B6-tTA) and allowed to explore for



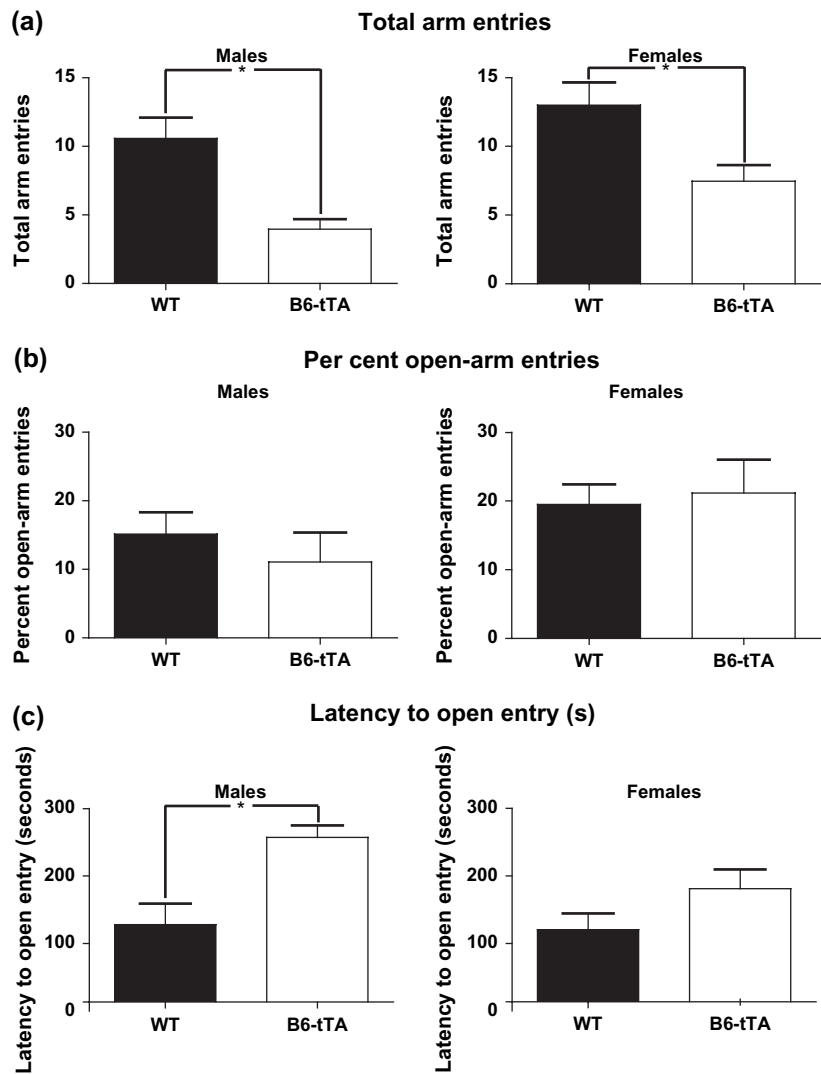
**Figure 1: CaMKII $\alpha$ -tTA mice exhibit decreased locomotor activity in an open field and light-dark box.** (a) B6-tTA mice of both sexes and F1-tTA mice traveled significantly less than their WT littermates during a 30-min (B6-tTA) or 5-min (F1-tTA) trial in an open field. (b) B6-tTA females and F1-tTA mice made significantly fewer transitions between sides of the light-dark box than their WT littermates during a 5-min (B6-tTA) or 10-min (F1-tTA) trial, \**P* < 0.05. All data are presented as mean  $\pm$  SEM.

a longer period (10 min, rather than 5 min for B6-tTA), F1-tTA mice exhibited a phenotype in the light–dark box similar to that of B6-tTA mice, that is, F1-tTA mice ( $n = 8$ ) transitioned fewer times between the compartments of the light–dark box when compared with their WT littermates ( $n = 12$ ). An unpaired  $t$ -test shows that F1-tTA mice made fewer transitions between the two sides of the light–dark box than their WT littermates (F1-tTA =  $23.13 \pm 4.60$  transitions and F1 WT =  $40.00 \pm 3.80$  transitions;  $t_{1,18} = 2.92$ ,  $P < 0.05$ ; Fig. 1 b); however, both genotypes spend a comparable percentage of the 10-min test period in the light side (F1-tTA =  $25.10 \pm 7.50\%$  and WT =  $23.70 \pm 3.00\%$ ;  $t_{1,18} = 0.188$ ,  $P > 0.05$ ; data not shown) as assessed by an unpaired  $t$ -test.

### CaMKII $\alpha$ -tTA mice exhibit decreased locomotor activity in the elevated plus maze and the elevated zero maze

Data from the elevated plus maze are shown in Fig. 2. Following placement into the center of the elevated plus

maze, both male and female B6-tTA mice ( $n = 20$  and  $n = 19$ , respectively) made fewer total entries into the arms of the plus maze (male B6-tTA =  $3.95 \pm 0.74$  entries and female B6-tTA =  $7.47 \pm 1.18$  entries) when compared with WT littermates ( $n = 19$ , entries =  $10.58 \pm 1.52$ ,  $t_{1,37} = 1.2$ ,  $P < 0.05$  for males;  $n = 18$ , entries =  $13.00 \pm 1.66$ ,  $t_{1,35} = 2.73$ ,  $P < 0.05$  for females; Fig. 2a). Neither male nor female B6-tTA mice differed from their WT littermates with respect to the percentage of total arm entries that were into open arms (B6-tTA =  $11.08 \pm 4.3\%$  and WT =  $15.13 \pm 3.2\%$ ,  $t_{1,37} = 0.7$ ,  $P > 0.05$  for males; B6-tTA =  $21.17 \pm 4.86\%$  and WT =  $19.52 \pm 2.92\%$ ,  $t_{1,35} = 0.29$ ,  $P > 0.05$  for females; Fig. 2b). Male B6-tTA mice did, however, take longer ( $257 \pm 18$  seconds) to make their first entry into an open arm than their WT littermates ( $128 \pm 31$  seconds;  $t_{1,37} = 3.6$ ,  $P < 0.05$ ; Fig. 2b). A similar trend exists for B6-tTA females, but this trend is not statistically significant (female B6-tTA =  $181.21 \pm 28.69$  seconds and female WT =  $120.33 \pm 25.17$ ;  $t_{1,35} = 1.59$ ,  $P > 0.05$ ; Fig. 2c).



**Figure 2: B6-tTA mice exhibit decreased locomotor activity in the elevated plus maze.** (a) B6-tTA mice of both sexes made fewer total entries into arms during a 5-min trial in the elevated plus maze. (b) Neither male nor female B6-tTA mice differed from their WT littermates with respect to per cent of total entries made into open arms. (c) B6-tTA males, but not B6-tTA females, exhibited a longer latency to their first entry into an open arm than their WT littermates,  $*P < 0.05$ . All data are presented as mean  $\pm$  SEM.

F1-tTA mice were not tested in the elevated plus maze, but rather in the related elevated zero maze. Data from the elevated zero maze are shown in Fig. 3. Following placement into one of the walled-in quadrants of the elevated zero maze, F1-tTA mice ( $n = 8$ ) exhibited a significantly increased latency to enter an open quadrant (130.8 seconds) when compared with their WT littermates ( $n = 12$ ; 5.1 seconds;  $t_{1,18} = 3.28$ ,  $P < 0.05$ ; Fig. 3a) as shown by an unpaired  $t$ -test. In fact, two F1-tTA mice failed to enter the open quadrant during the test session (300 seconds in duration). In addition, the results of an unpaired  $t$ -test shows that F1-tTA mice exhibited significantly less locomotor activity, as measured by total distance traveled, when compared with their WT littermates (F1-tTA =  $850.34 \pm 103.14$  cm vs. WT =  $1552.80 \pm 186.14$  cm;  $t_{1,18} = 2.87$ ,  $P < 0.05$ ; Fig. 3b). Finally, the percentage of total distance traveled in the open quadrants was shown by an unpaired  $t$ -test to be significantly shorter in F1-tTA mice ( $0.18 \pm 0.04$ ) compared with the WT mice ( $0.27 \pm 0.04$ ;  $t_{1,18} = 2.29$ ,  $P < 0.05$ ; Fig. 3c).

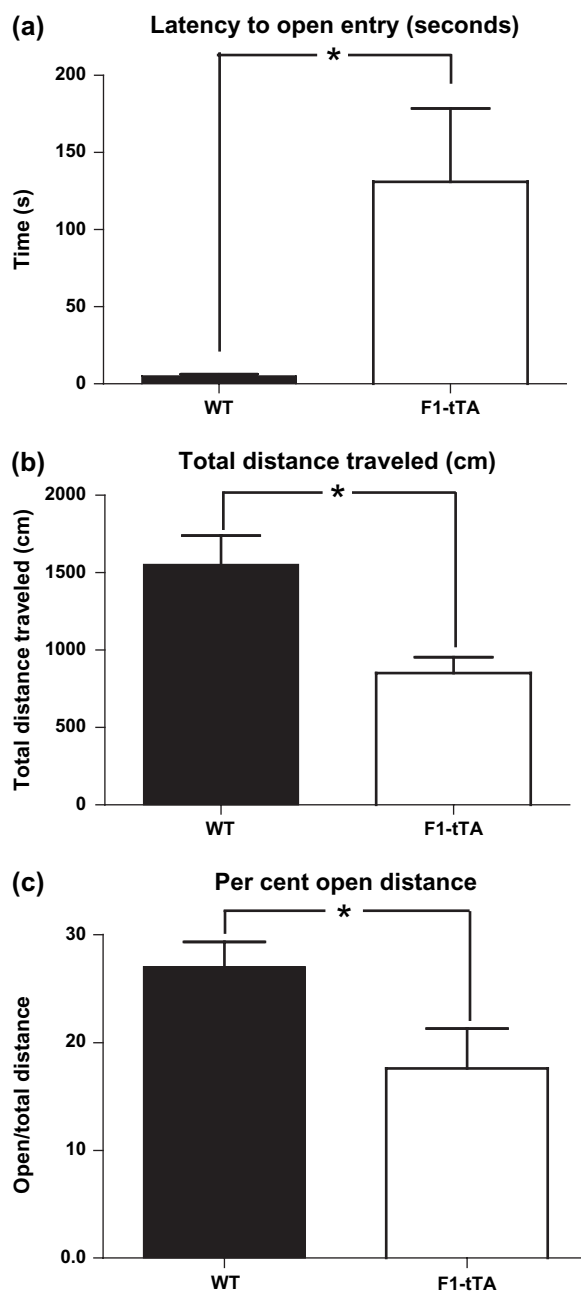
### B6-tTA, but not F1-tTA, mice exhibit modest impairments in motor coordination

Coordination and balance were first assessed with the accelerating rotarod. Data from the accelerating rotarod are presented in Fig. 4a,b. Performance on the accelerating rotarod was impaired in B6-tTA ( $n = 7$ ) compared with their WT littermate controls ( $n = 10$ ). Both genotypes improved performance across training days (Fig. 4a) as reflected in the significant effect of training day ( $F_{4,60} = 16.67$ ,  $P < 0.05$ ) as assessed by two-way repeated measures ANOVA. There was a significant effect of genotype ( $F_{4,15} = 6.79$ ,  $P < 0.05$ ) and a significant training day–genotype interaction ( $F_{4,15} = 2.53$ ,  $P < 0.05$ ). *Post hoc* analysis showed latency to fall off was shorter on the later trials (numbers 3–5) for B6-tTA mice compared with WT mice ( $P < 0.05$ ), while the initial performance was not statistically different between the two groups.

Accelerating rotarod data from F1-tTA are illustrated in Fig. 4b. Both genotypes ( $n = 18$  for F1-tTA and  $n = 25$  for WT) improved their performance across training days as reflected in the significant effect of training day ( $F_{4,164} = 88.95$ ,  $P < 0.05$ ) as assessed by a two-way repeated measures ANOVA. There was, however, no effect of genotype ( $F_{4,41} = 2.04$ ,  $P > 0.05$ ) or training day–genotype interaction ( $F_{4,164} = 0.90$ ,  $P > 0.05$ ).

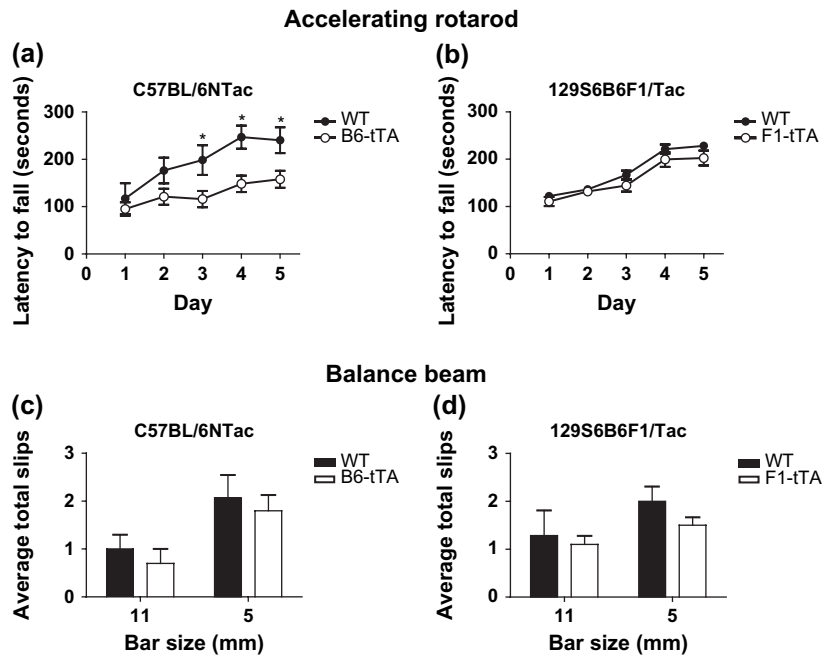
Motor coordination and balance was further examined using the balance beam task. As illustrated in Fig. 4c, performance on the balance beam did not differ between the B6-tTA ( $n = 7$ ) compared with their WT littermate controls ( $n = 10$ ). The number of hind foot slips did not differ between genotype for either the 11-mm beam ( $t_{1,15} = 0.39$ ,  $P > 0.05$ ) or 5-mm beam ( $t_{1,15} = 1.54$ ,  $P > 0.05$ ) as shown by unpaired  $t$ -tests.

Similar results were obtained when the F1-tTA animals were examined in the balance beams (Fig. 4d). Hind foot slips did not differ between genotype ( $n = 10$  for F1-tTA and  $n = 13$  for WT) for either the round 11 mm diameter beam ( $t_{1,21} = 0.70$ ,  $P > 0.05$ ) or square 5 mm wide beam ( $t_{1,21} = 0.45$ ,  $P > 0.05$ ) as shown by unpaired  $t$ -tests.



**Figure 3: F1-tTA mice exhibit decreased locomotor activity in the elevated zero maze.** (a) After initial placement in a walled quadrant of the elevated zero maze, F1-tTA mice took significantly longer to enter an unwalled quadrant than their WT littermates. (b) F1-tTA mice traveled significantly less than their WT littermates during a 5-min trial in the elevated zero maze. (c) Distance traveled in the unwalled quadrants as a percentage of total distance traveled in the elevated zero maze was significantly decreased in F1-tTA mice when compared with their WT littermates. \* $P < 0.05$ . All data are presented as mean  $\pm$  SEM.

**Figure 4: B6-tTA, but not F1-tTA mice exhibit modest impaired motor coordination and balance.** (a) Latency to fall from the accelerating rotarod is significantly shorter in B6-tTA mice than their WT littermates on days 3, 4 and 5 of training. (b) F1-tTA mice do not perform significantly different than their WT littermates on the accelerating rotarod. (c) The B6-tTA mice perform as well as their WT littermates on the balance beam task regardless of the beam diameter. (d) The F1-tTA mice exhibited the same average number of slips as their WT littermates when a 11-mm or 5-mm beam was used, \* $P < 0.05$ . All data are presented as mean  $\pm$  SEM.

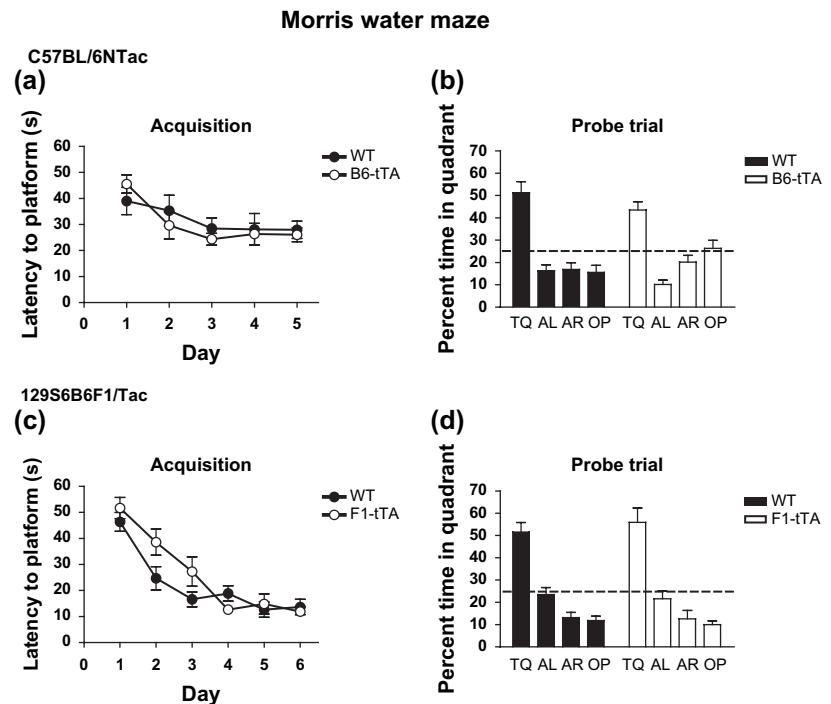


**Neither B6-tTA nor F1-tTA mice are impaired in two commonly used learning and memory paradigms**

Data from the hidden platform version of the MWM are shown in Fig. 5. Performance in the MWM did not significantly differ between B6-tTA ( $n = 10$ ) and WT ( $n = 7$ ) mice. During acquisition (Fig. 5a), both genotypes exhibited a decreased latency to reach the platform over the course

of training ( $F_{4,60} = 7.10, P < 0.0001$  effect of training). There was no effect of genotype on latency ( $F_{1,15} = 0.12, P > 0.05$ ) and no training day-genotype interaction ( $F_{4,60} = 91.36, P > 0.05$ ). During the probe trial (Fig. 5b), both B6-tTA and WT mice spent significantly more time in the training quadrant, the quadrant where the platform was previously located ( $t_{1,9} = 4.93, P < 0.05$  and  $t_{1,6} = 5.24, p < 0.05$ , respectively).

**Figure 5: Both B6-tTA and F1-tTA mice perform as well as their WT littermates in the MWM.** (a,c) C57BL/6NTac mice were trained for six trials a day for 5 days and 129SB6F1/Tac mice were trained for two trials a day for 6 days. The time to reach the hidden platform during training was not significantly different for B6-tTA or F1-tTA mice when compared with WT littermates. (b,d) A 60-second probe trial completed 24 h after the last training trial (day 6 for C57BL/6NTac mice; day 7 for 129SB6F1/Tac mice) shows that B6-tTA and F1-tTA mice and their WT littermates spend a significant amount of time during the trial searching in the quadrant where the platform was previously located (TQ; training quadrant), but there was no significant difference between the genotypes. The dashed line (25%) represents random or 'chance' performance, \* $P < 0.05$ . All data are presented as mean  $\pm$  SEM. AR, adjacent right; AL, adjacent left; OP, opposite.





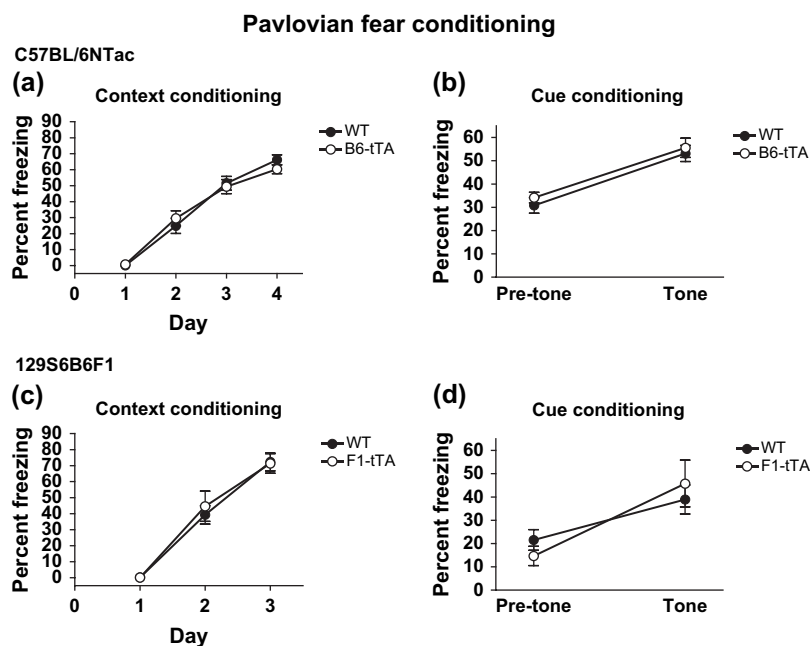
However, there was no significant difference in the amount of time that B6-tTA mice spent in the training quadrant compared with their WT littermates ( $t_{1,15} = 0.60$ ;  $P > 0.05$ ). In the visible platform version of the MWM, when the platform is marked with a distinct proximal cue (a flag), both groups found the platform with similar average latencies ( $t_{1,15} = 0.48$ ,  $P > 0.05$ ) and exhibited comparable average swim speeds ( $t_{1,15} = 0.85$ ,  $P > 0.05$ ) across the six trials as shown by unpaired  $t$ -tests (data not shown).

Similar to B6-tTA mice, F1-tTA mice did not differ from their WT littermates with respect to performance in the MWM. During acquisition (Fig. 5c), both genotypes ( $n = 8$  for F1-tTA and  $n = 12$  for WT) exhibited a decreased latency to reach the platform over the course of training ( $F_{5,90} = 29.15$ ,  $P < 0.05$  effect of training). Although the F1-tTA appear to exhibit greater latencies on days 2 and 3, there was no effect of genotype on latency ( $F_{1,18} = 3.37$ ,  $P > 0.05$ ) and no training day-genotype interaction ( $F_{5,90} = 2.10$ ,  $P > 0.05$ ) as assessed by a two-way repeated measures ANOVA. Data from a probe trial conducted 24 h after the last training trial on day 6 are shown in Fig. 5d. During the probe trial, both F1-tTA and WT mice spent significantly more time in the quadrant where the platform was previously located (training quadrant TQ in Fig. 5d) than would be predicted by chance ( $t_7 = 4.81$ ,  $P < 0.05$  and  $t_{11} = 6.30$ ,  $P < 0.05$ , respectively). However, there was no significant difference in the amount of time that F1-tTA mice spent in the training quadrant compared with their WT littermates ( $t_{1,18} = 0.60$ ,  $p > 0.05$ ). In the visible platform version of the MWM, both groups found the platform with similar average latencies ( $t_{1,18} = 0.023$ ,  $P > 0.05$ ) and exhibited comparable average swim speeds ( $t_{1,18} = 0.60$ ,  $P > 0.05$ ) across the six trials as shown by unpaired  $t$ -tests.

Data from Pavlovian fear conditioning are illustrated in Fig. 6. Prior to the first tone-shock pairing on day 1, neither

B6-tTA ( $n = 17$ ) nor WT ( $n = 18$ ) mice exhibited significant freezing (Fig. 6a). As training progressed, both genotypes showed significant increases in freezing as reflected in the effect of training day ( $F_{2,66} = 155.40$ ,  $P < 0.0001$ ); however, there was no effect of genotype ( $F_{1,33} = 0.07$ ;  $P > 0.05$ ) or training day-genotype interaction ( $F_{2,66} = 0.72$ ,  $P > 0.05$ ) as assessed by a two-way repeated measures ANOVA. After completion of training, exposure to the context alone on day 4 produced similar degrees of freezing in both genotypes (B6-tTA = 60.32 and WT = 66.22;  $t_{1,33} = 1.38$ ;  $P > 0.05$ ) as shown by an unpaired  $t$ -test. On day 5, the chambers were reconfigured. After 2 min of baseline, the tone used during conditioning was delivered for 3 min (Fig. 6b). During the tone, freezing increased significantly (as shown by paired  $t$ -tests) over baseline in both B6-tTA ( $t_{1,16} = 5.95$ ;  $P < 0.0001$ ) and WT ( $t_{1,17} = 9.12$ ,  $P < 0.0001$ ) mice suggesting that both groups learned the tone-shock association. However, there was no effect of genotype as the genotypes froze to a similar degree both before the tone (B6-tTA = 34.17 and WT = 30.80;  $t_{1,33} = 0.83$ ;  $p > 0.05$ ) and during the tone [B6-tTA = 55.57 and WT = 53.09;  $t_{1,33} = 0.46$ ;  $P > 0.05$ ] as shown by unpaired  $t$ -tests.

Like B6-tTA mice, F1-tTA mice do not differ from their WT littermates with respect to performance in the Pavlovian fear conditioning paradigm. Prior to the first tone-shock pairing on day 1, neither F1-tTA ( $n = 8$ ) nor WT ( $n = 12$ ) mice exhibited significant freezing (Fig. 6c). As training progressed, both genotypes showed significant increases in freezing as reflected in the effect of training day ( $F_{1,18} = 65.50$ ;  $P < 0.05$ ; Fig. 6c); however, there was no effect of genotype ( $F_{1,18} = 0.80$ ;  $P > 0.05$ ; Fig. 6c) or training day-genotype interaction ( $F_{1,18} = 0.28$ ;  $P > 0.05$ ; Fig. 6c) as assessed by a two-way repeated measures ANOVA. Exposure to the context alone (in the absence of tone) on day 3 produced similar degrees of freezing in both F1-tTA and WT mice (F1-tTA =



**Figure 6: Both B6-tTA and F1-tTA mice exhibit normal Pavlovian fear conditioning.** (a,c) Both B6-tTA and F1-tTA mice exhibit similar levels of freezing to context as their WT littermates prior to conditioning (day 1), 24 h after the first conditioning trial (day 2), 24 h after the second conditioning trial (day 3) and 24 h after the third conditioning trial (day 4; B6 mice only). (b,d) B6-tTA and F1-tTA mice exhibit similar levels of generalized freezing as their WT littermates upon placement in a reconfigured context (Pre-tone) as well as equivalent freezing to tone presentation (Tone). \* $P < 0.05$ . All data are presented as mean  $\pm$  SEM.



71.38 and WT = 72.33;  $t_{1,18} = 11$ ;  $P > 0.05$ ; Fig. 6c) as shown by an unpaired  $t$ -test. On the day after the context test (day 4), the chambers were reconfigured and mice were placed in the chambers for 5 min. After 2 min of baseline, the tone used during conditioning was delivered for 3 min (Fig. 6d). During the tone, freezing increased significantly (as shown by paired  $t$ -tests) over baseline in both F1-tTA ( $t_{1,18} = 3.84$ ;  $P < 0.05$ ) and WT ( $t_{1,18} = 3.75$ ;  $P < 0.05$ ) mice suggesting that both groups learned the tone–shock association. However, there was no effect of genotype as the genotypes froze to a similar degree both before the tone (F1-tTA = 14.68 and WT = 21.54;  $t_{1,18} = 1.07$ ;  $p > 0.05$ ) and during the tone (F1-tTA = 45.76 and WT = 38.91;  $t_{1,18} = 0.61$ ;  $P > 0.05$ ) as shown by unpaired  $t$ -tests.

## Discussion

The principal findings presented here are that CaMKII $\alpha$ -tTA mice exhibit decreased locomotor activity compared with their WT littermates. This conclusion is based on the observation that locomotion in a number of behavioral paradigms including the open-field test, light–dark box, elevated plus maze and elevated zero maze are consistently lower in CaMKII $\alpha$ -tTA mice than WT mice. In light of the fact that decreased locomotor activity in these paradigms often reflects increased anxiety rather than a primary effect on locomotion, it is interesting to note that measures of anxiety that are less dependent on total locomotor activity are not consistently affected in CaMKII $\alpha$ -tTA mice. For example, CaMKII $\alpha$ -tTA mice make fewer transitions in the light–dark box, but spend a similar percentage of time exploring the light side when compared with WT littermates. In addition, B6-tTA mice make fewer total entries into arms of the elevated plus maze, but do not differ from their WT littermates with respect to the percentage of total entries made into an open arm. Together, these observations suggest that the decreased locomotor activity observed in CaMKII $\alpha$ -tTA mice is a primary effect on locomotion and not secondary to anxiety.

These studies also show that CaMKII $\alpha$ -tTA mice perform as well as WT mice during Pavlovian fear conditioning and in the MWM, two commonly used paradigms in the study of learning and memory – an important finding given the extensive use of CaMKII $\alpha$ -tTA mice in the investigation of the molecular genetics of learning and memory (Bejar *et al.* 2002; Isiegas *et al.* 2006) and models of memory-related pathologies (Ramsden *et al.* 2005).

Motor coordination and balance were also investigated in CaMKII $\alpha$ -tTA mice using the accelerating rotarod, but the data are inconsistent: B6-tTA mice exhibit modest impairments in performance on the rotarod, particularly on the later training days, whereas F1-tTA mice do not. This inconsistency with regard to the rotarod likely reflects the impact of genetic background on performance in this task. These results are consistent with previous reports showing that genetic background can alter phenotypic expression in transgenic mice (Dobkin *et al.* 2000; McKinney *et al.* 2005; Murphy *et al.* 2004; Wolfer *et al.* 1997). In addition to being limited to the later training trials on the rotarod, the motor impairments observed in the B6-tTA mice did not generalize to balance beam task,

suggesting that the B6-tTA mice exhibit a fairly selectively impairment in motor coordination.

The most striking aspect of the abnormal behavioral phenotype observed in CaMKII $\alpha$ -tTA mice is its robustness in the face of widely varying environmental conditions and biological factors. Because the data presented here are the product of two studies designed and executed by two different, independent groups at the University of Michigan and brought together *post hoc*, the housing and testing conditions are quite different. Environmental factors such as housing conditions (Lewejohann *et al.* 2006), testing procedures (Crawley 1999) and even laboratory or experimenter (Crabbe *et al.* 1999; Wahlsten *et al.* 2006) can greatly impact the results of mouse behavior studies. It is conceivable that some of the phenotypic differences observed in genetically modified mice, especially subtle ones, might be obscured by the effects of environmental factors. In addition to the different environmental conditions, there were different biological factors at play in the two studies. For example, one group performed their experiments on mice with a C57BL/6NTac genetic background and the other used mice with a 129S6B6F1/Tac genetic background (some of the experiments were also performed on mice with a C57BL/6J genetic background; see Supplementary material, Figs S1, S2). For most of the experiments, the male and female C57BL/6NTac mice were analyzed separately, whereas sexes were combined in the 129S6B6F1/Tac study. Genetic background and sex dramatically influence mouse behavior (Palanza 2001; Voikar *et al.* 2001; Wolfer *et al.* 2002) and are known to modify the phenotypes of genetic mouse mutants (Buchner *et al.* 2003; Graves *et al.* 2002; Hayward & Low 2007; Ris *et al.* 2005). The fact that the abnormal behavioral phenotype observed in CaMKII $\alpha$ -tTA mice is generally resistant to such dramatic differences in study design and execution suggests that these observations have significant implications for studies using this particular line of CaMKII $\alpha$ -tTA mice under a wide range of conditions.

The observation that CaMKII $\alpha$ -tTA mice exhibit decreased locomotor activity was consistent across both of the studies presented here, but does not agree with other published accounts of locomotor activity in these mice. For example, Gross *et al.* (2002) reported that CaMKII $\alpha$ -tTA mice did not differ from WT controls in a number of behavioral paradigms including open-field test and elevated plus maze. Perhaps, the most striking difference between that study and the ones presented here is the difference in genetic background. The mice used in Gross *et al.* (2002) were on a mixed genetic background generated from 129/Sv, C57BL/6J and CBA/J strains. It is not clear what the relative contributions of each of these different strains were to the mice used in that study, but the mere contribution of the CBA/J strain to the hybrid background makes the strain of mice used in that study dramatically different from either of the genetic backgrounds tested in the present study.

The exact biological mechanism or mechanisms for the observed behavioral abnormalities remains unknown. One possible mechanism is that the transgene may have integrated into a locus that is important for normal locomotor activity and/or motor performance rendering that locus non-functional or differently functional. Certainly, novel genes have

been identified as a result of transgene insertional mutations (Burgess *et al.* 1995; Kohrman *et al.* 1995). Another possible mechanism is that tTA expression is somehow toxic, which in turn leads to the abnormal behavior. Consistent with this idea, it has previously been reported that the high levels of tTA expression obtained using the human glial fibrillary acidic protein promoter in an autoregulatory tetracycline-regulated system leads to cerebellar atrophy and ataxia in transgenic rats (Barton *et al.* 2002). It seems likely that the ataxia and cerebellar atrophy are the result of tTA toxicity because administration of doxycycline – which in the autoregulatory system blocks the expression of tTA – prevented both the atrophy and ataxia (Barton *et al.* 2002). Similarly, it has been shown that cardiac-specific expression of tTA driven by the  $\alpha$ -myosin heavy chain promoter ( $\alpha$ -MHC-tTA mice) resulted in cardiomyopathy (McCloskey *et al.* 2005). Finally, it has recently been reported that mice expressing a modified version of tTA, the reverse tetracycline activator (i.e. rTA), in lung tissue exhibited an emphysema-like phenotype suggesting that expression of this related protein is toxic to pneumocytes (Sisson *et al.* 2006).

One limitation in interpreting the above studies as well as the studies presented here is that in each case the mice under investigation in each study were the product of a single founder line. Therefore, at present, it is not possible to determine the exact biological mechanism that gives rise to the observed behavioral phenotype in the CaMKII $\alpha$ -tTA mice. Thus, further studies employing alternate lines of CaMKII $\alpha$ -tTA mice will be necessary to elucidate the mechanism of the abnormal behavioral phenotype described here.

The findings presented here highlight the importance of appropriate controls when using tetracycline-regulated gene expression systems and, in particular, when using CaMKII $\alpha$ -tTA mice as part of the study design. Obviously, the most conservative approach would be to analyze and present the data from all four possible genotypes: mice bigenic for CaMKII $\alpha$ -tTA and TRE-coupled transgenes, mice transgenic for either the CaMKII $\alpha$ -tTA or the TRE-coupled transgene, and non-transgenic WT mice that allows for the isolation of effects of both of the transgenes alone and permits the study of regulated expression of the TRE-coupled transgene. An alternative approach is to use mice bigenic for CaMKII $\alpha$ -tTA and the TRE-coupled transgenes and compare the behavior in mice treated with tetracycline or doxycycline with bigenic mice that do not receive the drug. This strategy would allow the effect of regulated expression of the TRE-coupled transgene to be studied, but would not isolate the effect of each transgene independently. As any phenotype caused by either of the transgenes alone may obscure the effects of regulated expression of the TRE-coupled transgene, this design increases the chance of false-negative results and decreases the ability of one to accept the null hypothesis. This type of strategy may best be used in studies where an effect of each of the transgenes alone has been established for a particular paradigm. For example, the studies presented here failed to find any significant alteration in performance in the MWM and Pavlovian fear conditioning paradigms and therefore suggest that the use of the CaMKII $\alpha$ -tTA in these paradigms is appropriate. However, even when these paradigms are used, the effect of each new TRE-coupled transgene alone needs to

be studied before this strategy can effectively be employed. Regardless of the strategy employed, the use of CaMKII $\alpha$ -tTA mice to study locomotor activity or behaviors dependent on normal locomotor activity, including numerous tests of anxiety-like behavior, should be approached with caution given the profound impairment of both B6-tTA and F1-tTA mice in such paradigms.

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## Supplementary material

The following supplementary material is available for this article:

**Figure S1:** B6-tTA (Jax) mice exhibited decreased locomotor activity in the open-field test and light-dark box.

**Figure S2:** B6-tTA (Jax) mice exhibited decreased locomotor activity in the elevated plus-maze.

**Figure S3:** Data from the elevated zero maze reanalyzed for sex genotype interaction.

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