



Research Paper

MARCKSL1 Regulates Spine Formation in the Amygdala and Controls the Hypothalamic–Pituitary–Adrenal Axis and Anxiety-Like Behaviors

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ARTICLE INFO

Article history:

Received 26 September 2017

Received in revised form 16 March 2018

Accepted 16 March 2018

Available online 19 March 2018

Keywords:

MARCKSL1

Spine formation

Amygdala

HPA axis

Anxiety-like behaviors

ABSTRACT

Abnormalities in limbic neural circuits have been implicated in the onset of anxiety disorders. However, the molecular pathogenesis underlying anxiety disorders remains poorly elucidated. Here, we demonstrate that myristoylated alanine-rich C-kinase substrate like 1 (MARCKSL1) regulates amygdala circuitry to control the activity of the hypothalamic–pituitary–adrenal (HPA) axis, as well as induces anxiety-like behaviors in mice. MARCKSL1 expression was predominantly localized in the prefrontal cortex (PFC), hypothalamus, hippocampus, and amygdala of the adult mouse brain. MARCKSL1 transgenic (Tg) mice exhibited anxiety-like behaviors dependent on corticotropin-releasing hormone. MARCKSL1 increased spine formation in the central amygdala, and downregulation of MARCKSL1 in the amygdala normalized both increased HPA axis activity and elevated anxiety-like behaviors in Tg mice. Furthermore, MARCKSL1 expression was increased in the PFC and amygdala in a brain injury model associated with anxiety-like behaviors. Our findings suggest that MARCKSL1 expression in the amygdala plays an important role in anxiety-like behaviors.

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1. Introduction

Anxiety disorders have a reported lifetime prevalence of approximately 14–29% worldwide (Kessler et al., 2005). Patients with anxiety disorders frequently present with comorbid psychiatric disorders, such as major depressive disorder (Gorman, 1996–1997; Ressler and Mayberg, 2007). Understanding the neuronal and molecular mechanisms that trigger anxiety disorders is essential for developing effective therapeutic strategies.

The limbic-cortical system is the principal neural circuit that is dysregulated and involved in the onset of psychiatric disorders, including mood and anxiety disorders (Craske et al., 2009; Drevets, 2000; Ressler and Mayberg, 2007). It has been demonstrated that the

prefrontal cortex (PFC), hippocampus, and amygdala are the main brain areas dysregulated in these emotional disorders (Davidson, 2002; Drevets, 2000; Ressler and Nemeroff, 2000; Roozendaal et al., 2009; Stein and Stein, 2008). In particular, the neural functioning of the amygdala plays a pivotal role in the response to stress exposure (Allen and Allen, 1974; Roozendaal et al., 2009), as well as in the onset of anxiety disorders (Etkin et al., 2009; Lesscher et al., 2008; Lyons and Thiele, 2010; Shekhar et al., 2005; Tye et al., 2011). However, the molecular pathogenesis underlying anxiety disorders that evoke circuit dysregulation remains largely unknown.

Myristoylated alanine-rich C-kinase substrate like 1 (MARCKSL1) is a member of the MARCKS family, a group of acidic proteins localized to the plasma membrane. Microarray analysis suggested that MARCKSL1 is expressed during development of the mouse PFC, and is therefore a molecular candidate for neural development (Semeralul et al., 2006). MARCKSL1 is known to be a primary substrate of protein kinase C (PKC), and it regulates membrane-cytoskeletal plasticity by altering the actin cytoskeleton (Arbuzova et al., 2002; Björkblom et al., 2012; Sundaram et al., 2004). A previous study indicated that MARCKSL1 regulates neuronal filopodia formation and bundle F-actin (Björkblom et al., 2012). As postnatal neurodevelopmental processes include various structural changes that mediate synaptic formation and pruning, as well as dendritic and axonal growth (Webb et al.,

Abbreviations: PFC, prefrontal cortex; MARCKSL1, myristoylated alanine-rich C-kinase substrate like 1; PKC, protein kinase C; Tg, transgenic; WT, wild-type; CRH, corticotropin-releasing hormone; CRHR, corticotropin-releasing hormone receptor; DMSO, dimethylsulfoxide; siRNA, small interfering RNA; CeA, central amygdala; PVN, paraventricular hypothalamic nucleus; LA, lateral amygdala; BLA, basolateral amygdala; HPA, hypothalamic–pituitary–adrenal; BNST, bed nucleus of the stria terminalis; JNK, Jun N-terminal protein kinase.

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2001), the molecular features of MARCKSL1 imply an important function in structural maintenance and neuronal plasticity. However, the precise physiological role of MARCKSL1 in the adult brain and its subsequent effects on behaviors remain largely unclear. Although MARCKSL1 is predominantly expressed in the immature brain, it remains localized in adult brain regions associated with emotional processing, including the PFC, hippocampus, and amygdala (McNamara and Lenox, 1998). Considering MARCKSL1 expression in the adult limbic system and its putative role in altering neuronal plasticity and circuitry, we believed that functional analysis of MARCKSL1 in vivo would facilitate our understanding of the molecular mechanisms that control emotional processes.

Here, using overexpression and knockdown techniques in vivo, we analyzed the role of MARCKSL1 in brain function, focusing on mood and anxiety. We demonstrate a clear association between MARCKSL1 expression in neural circuitry and generation of anxiety-like behaviors.

2. Materials and Methods

2.1. Generation of the Transgenic Mouse Model

The MARCKSL1 locus was amplified from the genome of an adult (12-week-old) mouse brain via PCR with KOD FX Neo (TOYOBO). A 0.6 kb fragment was identified that hybridized to the mouse *Marcks11* cDNA. This fragment was isolated with 1.5% agarose gel electrophoresis, and further purified with Wizard® SV Gel and PCR Clean-up System (Promega Corporation). The fragment was subcloned into the *XhoI* and *EcoRI* site of a pCAGGS vector (Addgene) by introducing *Marcks11* containing a HA tag (Fig. 2A). The amplified DNA plasmid was treated with *BamHI*, *ScaI*, and *Sall* to prepare a 3.1 kb fragment, which was microinjected into the pronucleus of fertilized B6D2F1 mouse eggs. Male and female mice expressing the *Marcks11* transgene were identified with Southern blotting. Heterozygous offspring were mated to produce homozygous mutant animals. Genotypes at the pCAGGS-*Marcks11* locus were determined with PCR.

For genotyping of mice, DNA was prepared from mouse tails. PCR amplifications were performed through 35 cycles of denaturation at 98 °C, annealing at 55 °C, and extension at 68 °C, followed by a final extension at 68 °C. The following primers were used: 5'-CTTCCTTGTCCCAATCTGTGCGGAGCCGAAATCT-3' and 5'-GGGTTAAGTCTCCATTGCTTCTCACGTGGCCATTCT-3'. PCR products were electrophoresed in Tris-acetate-EDTA buffer containing 1% agarose (Nippon Gene) and visualized by staining with ethidium bromide. Procedures related to the generation of the transgenic (Tg) mice were approved by the animal ethics committee of Kindai University.

2.2. Animals

The mice were bred and maintained in a specific pathogen-free environment in the Institute of Experimental Animal Sciences, Kindai University Medical School. Eight-week-old C57BL/6 N male mice were purchased from Japan SLC. MARCKSL1 Tg C57BL/6N mice were obtained by backcrossing MARCKSL1 Tg B6D2F1 to C57BL/6N strain for at least five generations. Mice used in these studies were propagated by mating +/Tg zygotes, thereby producing +/+, +/Tg, and Tg/Tg offspring. The +/+ mice were used as wild-type (WT) controls. Three or four mice were each housed in a standard cage on a 12 h light/dark cycle, with food and water available ad libitum. Total numbers of male mice used in this study were as follows: 9–12-week-old WT (n = 71), +/Tg (n = 11), and Tg/Tg mice (n = 57). The experiments were conducted in accordance with the guidelines for the care and use of laboratory animals, and were approved by the institutional committee of Kindai University and Kanazawa Medical University.

2.3. Drug Treatment

Antalarmin, a corticotropin-releasing hormone (CRH) receptor 1 (CRHR1) antagonist (Sigma-Aldrich, A8727), was dissolved in 4% dimethylsulfoxide (DMSO) with 0.9% saline as a stock solution (5 mg/ml). It was brought to a final concentration of 2 mg/ml with 4% DMSO immediately before the experiment. Mice received intraperitoneal injections of 4% DMSO control vehicle (WT, n = 7; Tg/Tg, n = 7) or antalarmin (10 mg/kg; WT, n = 5; Tg/Tg, n = 5) 40 min before behavioral testing.

2.4. Small Interfering RNA (siRNA) Transfection In Vivo

For knockdown experiments, we used *Marcks11* siRNA (Sigma-Aldrich) containing the following sequences: sense (5'-CAUCAGCCAUUUGGUCUUATT-3') and antisense (5'-UAAGACCAAAUGGCCUGAUGTT-3'). *Marcks11* siRNA (WT, n = 3; Tg/Tg, n = 9) or scrambled siRNA (WT, n = 9; Tg/Tg, n = 7) was injected intracranially into the bilateral central amygdala (CeA) of mice following the procedures in a previous report (Lyons and Thiele, 2010), with i-Fect™ transfection reagents (NeuroMics). Mice injected with the scrambled siRNA were used as controls. Mice were anesthetized and placed in a stereotaxic frame. The skull overlying the right and left primary sensory cortex was carefully removed with a drill, and then *Marcks11* or scrambled siRNA with i-Fect reagent was injected at two sites on each side within the CeA (coordinates from bregma: 1.2 mm posterior/2.9 mm lateral, 1.7 mm posterior/2.9 mm lateral, all at 4.2 mm depth, 0.3 µl/site) with a glass capillary (tip diameter, 80 µm). Mice were sacrificed 9–10 days after intracranial injections.

2.5. Brain Injury Models

The mice were placed in a stereotaxic frame (Narishige) after deep anesthesia (Tanaka et al., 2013). The skull was exposed with a midline skin incision, and skull bone overlying the left motor cortex was removed. Hemicortical lesions (left side, depth: 1.0 mm in the motor cortex) were induced in mice by using cortical ablation with a pipette (n = 11) (Omoto et al., 2010; Tanaka et al., 2013). The injured area consisted of primary and secondary motor areas of the cerebral cortex (Tennant et al., 2011). Thereafter, the skull was replaced, the wound was sutured, and the mice were housed in standard cages with free access to food and water. Intact mice (n = 9) were used as controls.

2.6. Behavioral Tests

All behavioral tests were performed between 9 and 11 a.m. with male mice (10–12-week-old). After each test, all testing apparatus was cleaned with 0.5% hibitane (Sumitomo Dainippon Pharma) in water to prevent potential bias based on olfactory cues.

2.6.1. Light/Dark Transition Test

The light/dark transition test was conducted with reference to previous reports (Kim et al., 2013; Takao and Miyakawa, 2006). The apparatus used for the light/dark transition test consisted of a cage (30 cm × 30 cm × 30 cm) divided into two sections: a light and a dark chamber. Mice were placed in the dark chamber and allowed to move freely between the two chambers for 10 min. The total number of transitions between chambers, the time spent in the light chamber, and the latency to the first entrance into the light chamber were recorded.

2.6.2. Elevated Plus Maze

The elevated plus maze was conducted with reference to previous reports (Ihara et al., 2007; Komada et al., 2008). The elevated plus maze consists of two open arms and two enclosed arms (30 cm × 6 cm) with 20 cm high walls. The arms and central square were made of gray plastic

plates and were elevated 41 cm from the floor. Arms of the same type were arranged opposite each other. Mice were placed in the central area of the apparatus (6 cm × 6 cm) facing the enclosed arms. The number of total entries, the percentage of open arm entries, and the time spent in the open arms were recorded for 10 min.

2.7. Statistical Analysis

All data are presented as mean ± SEM. Statistical analyses were performed with Statcel2 software. Differences between experimental group pairs were analyzed with Student's *t*-tests. Differences among the groups were analyzed by one-way ANOVA followed by the Tukey-Kramer tests. *P* values < 0.05 were considered statistically significant.

Additional experimental procedures are available in the Supplemental Experimental Procedures.

3. Results

3.1. MARCKSL1 Expression in Emotion-Related Regions of the Adult Brain

First, we investigated MARCKSL1 expression in the adult brain of WT mice, focusing on several regions associated with emotional processing, including the PFC, hypothalamus, hippocampus, and amygdala. As previously reported, we showed expression of MARCKSL1 in the dentate gyrus of the hippocampus (McNamara and Lenox, 1998). In addition, we found clear expression in the PFC, paraventricular hypothalamic nucleus (PVN), and amygdala (Fig. 1A–D and Supplementary Fig. 1A).

MARCKSL1 expression varied among amygdala subregions, with abundant expression observed in the lateral amygdala (LA), but low expression observed in the CeA and basolateral amygdala (BLA) (Fig. 1D). MARCKSL1 was mainly located in the dendrites and plasma membrane of neurons, although some expression was observed in axons, illustrated by the pattern of expression in mossy fibers of the hippocampus (Fig. 1A–D and Supplementary Fig. 1B).

We further examined whether MARCKSL1 was expressed in glial cells. We observed MARCKSL1 expression in oligodendrocytes (Supplementary Fig. 1C), but not in astrocytes (Supplementary Fig. 1D) or microglia (Supplementary Fig. 1E). The pattern of expression within neural circuitry associated with emotion regulation suggests that MARCKSL1 expression may be associated with emotional behavior in mice.

3.2. Generation of MARCKSL1 Tg Mice and Anxiety-Like Behavioral Characterization

To elucidate the role of MARCKSL1 in emotional behavior, we generated Tg mice that constitutively and ubiquitously overexpressed MARCKSL1 with the CAG promoter (Fig. 2A). We obtained MARCKSL1 Tg mice lines using genotyping primers via the PCR method (Fig. 2A). In the 12-week-old brain, MARCKSL1 Tg homozygous mice expressed approximately five times more *Marcksl1* mRNA compared with WT mice (Supplementary Fig. 2A). Consistent with mRNA expression, western blotting for MARCKSL1 proteins in the brains of Tg/Tg mice showed higher levels of expression compared with those of WT and +/Tg mice (Supplementary Fig. 2B and C). In situ hybridization for *Marcksl1* mRNA further demonstrated that *Marcksl1* mRNA expression was induced in all brain regions in adult Tg/Tg mice (Supplementary Fig. 2D and E). In situ hybridization and immunohistochemical analysis confirmed this observation, revealing marked increases in the expression of MARCKSL1 protein and mRNA in the BLA and CeA of Tg/Tg mice (Fig. 2B and C).

Gross appearance was comparable among the genotypes. There were no significant differences in body weight or brain weight among WT, +/Tg and Tg/Tg mice (Supplementary Fig. 2F and G). In addition, compared with WT mice, both +/Tg and Tg/Tg mice showed no difference in brain morphology (Supplementary Fig. 2H).

We next examined anxiety-related behavior in Tg/Tg mice. In the light/dark transition test, Tg/Tg mice exhibited significantly longer latencies to enter the light box, decreased numbers of transitions, and decreased time spent in the light box (Fig. 2D). In the elevated plus maze, Tg/Tg mice showed significantly fewer total arm entries, a decreased duration of time spent in the open arms and a lower percentage of open arm entries (Fig. 2E). Moreover, compared with WT mice, Tg/Tg mice spent less time in the center area of the open field in the open field test (Supplementary Fig. 2I), and showed a trend towards a longer time spent immobile in the forced swim test (Supplementary Fig. 2J). In contrast, in the social behavioral test there was no difference between WT and Tg/Tg mice (Supplementary Fig. 2K). Collectively, the results indicate that MARCKSL1 Tg mice exhibited anxiety-like behaviors.

3.3. Anxiety-Like Behaviors in MARCKSL1 Tg Mice Depend on CRH Production

We further tested the effects of activation of the hypothalamic-pituitary-adrenal (HPA) axis under basal conditions in MARCKSL1 Tg mice. The HPA axis is the endocrine core of the stress system, which involves hypothalamic CRH, pituitary corticotropin, and adrenal corticosterone (de Kloet et al., 2005). CRH is produced in the PVN (Smith and Vale, 2006), which is the primary driver of the HPA axis. We therefore examined neural activity in CRH neurons in the PVN of MARCKSL1 Tg mice by measuring c-Fos expression levels with immunohistochemical analysis (Fig. 3A).

The number of CRH and c-Fos double-positive cells in the PVN was significantly increased in MARCKSL1 Tg mice (Fig. 3B). Furthermore, *Crh* mRNA levels in the hypothalamus (Fig. 3C) and plasma corticosterone concentration (Fig. 3D) was significantly increased in Tg/Tg mice. We further examined expression of *Crh* and stress-related receptor mRNAs (*Crhr1*, *Crhr2*, *Nr3c1*, and *Nr3c2*) in the amygdala, hypothalamus, and hippocampus. *Nr3c1* (also known as glucocorticoid receptor) and *Nr3c2* (also known as mineralocorticoid receptor) are receptors for corticosterone and cortisol, and are widely distributed in the brain (de Kloet et al., 2008; Morimoto et al., 1996; Reul and de Kloet, 1985). There were no differences in the expression of these receptors between WT and Tg/Tg mice (Supplementary Fig. 3A–C). These data suggest that MARCKSL1 Tg mice have a dramatic over-activation of the HPA axis, similar to that observed in mice exposed to chronic stress, which may result in anxiety phenotypes.

We next investigated whether anxiety-like behavior in MARCKSL1 Tg mice depended on CRH levels. Tg/Tg mice were injected with 10 mg/kg antalarmin (CRHR1 antagonist; i.p.) or DMSO control vehicle 40 min prior to behavioral testing (Fig. 3E). Control Tg/Tg mice injected with vehicle exhibited anxiety-like behaviors. However, antalarmin-injected Tg/Tg mice showed near normalized behavior (similar to that of WT mice) in the light/dark transition test (Fig. 3F). These results indicate that MARCKSL1 Tg mice show elevated anxiety-like behaviors in a CRHR1 dependent manner.

3.4. Upregulation of MARCKSL1 Increases the Dendritic Spine Density of Amygdala Spiny Neurons

Previous reports have indicated that projection neurons to the hypothalamus originate predominantly in the amygdala and thus that this area may be functionally important in the onset of anxiety disorders (Kalin et al., 2004; LeDoux et al., 1988). To investigate the mechanisms underlying CRH-dependent anxiety-like behaviors in MARCKSL1 Tg mice, we examined dendritic spine density in amygdala neurons with the Golgi-stain method. Dendritic spine densities were assessed in the CeA, which mostly contains GABAergic spiny neurons (McDonald, 1982a; Sun and Cassell, 1993) (Fig. 4A), and the BLA and LA, which are predominantly composed of excitatory neurons (Carlsen, 1988; McDonald, 1982b, 1984) (Supplementary Fig. 4A and E). The neurons

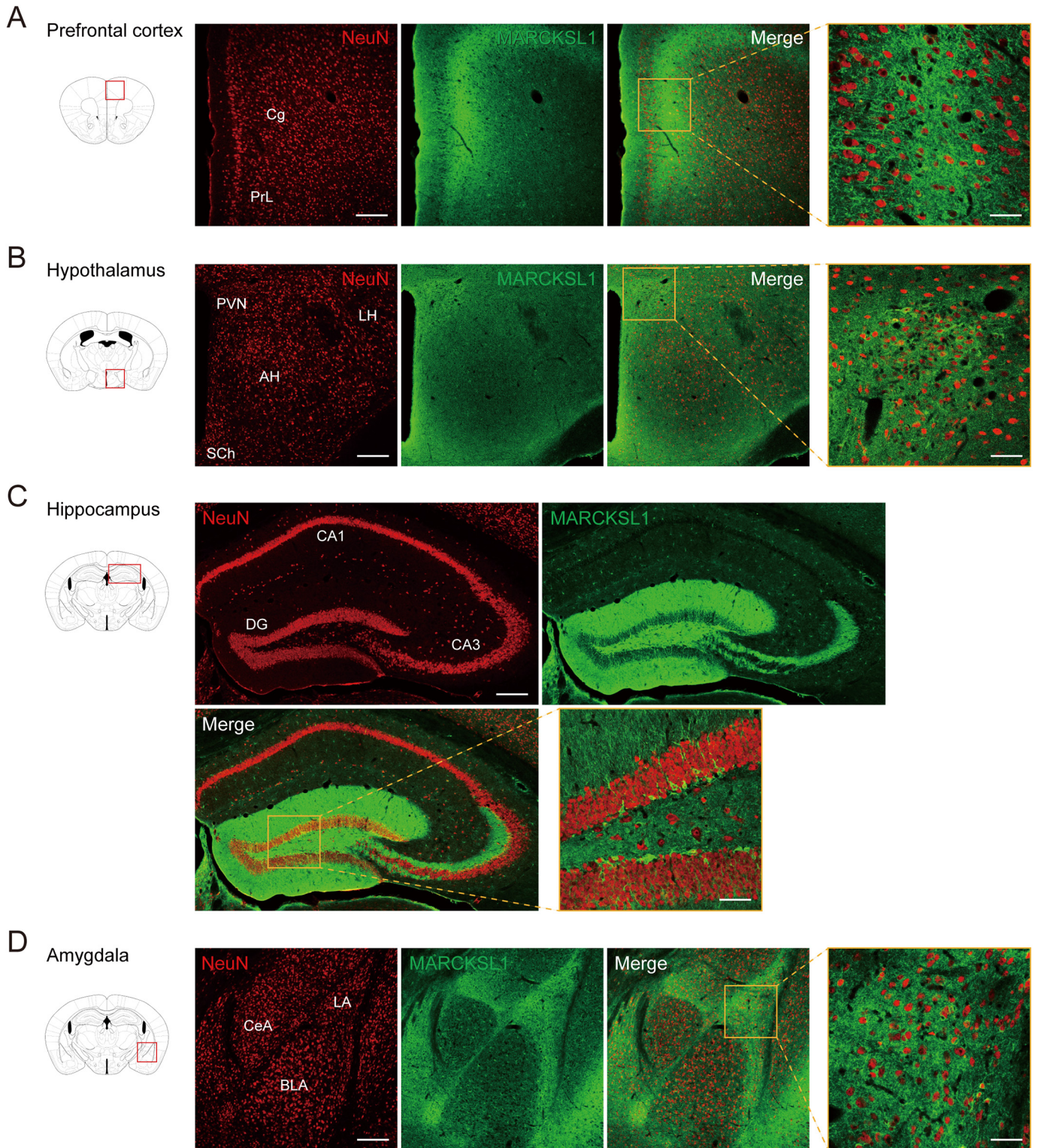


Fig. 1. MARCKSL1 is expressed in adult brain regions associated with emotional processing. (A–D) MARCKSL1 (green) and NeuN (red) staining in the: (A) PFC (Cg, cingulate cortex; PrL, prelimbic cortex); (B) hypothalamus (AH, anterior hypothalamic area; LH, lateral hypothalamic area; Sch, suprachiasmatic nucleus); (C) hippocampus (CA1, cornu ammonis 1; CA3, cornu ammonis 3); and (D) amygdala. The pictures on the *right* are the magnified views of the squares in the *left* panels. Scale bars: A–D, (*left*) 200 μ m, (*right*) 50 μ m.

were visualized with a Golgi stain, and dendritic spines were quantified by counting the number of spines at each 10 μ m from the cell soma.

The spine density of spiny neurons of the CeA (Fig. 4B–D) and BLA (Supplementary Fig. 4B–D) were significantly increased in Tg/Tg mice. These data indicate that MARCKSL1 upregulation induced extensive

spinogenesis of spiny neurons in the CeA and BLA. We further analyzed the spines of Golgi-stained pyramidal neurons from the BLA and LA by quantifying the number of spines per 10 μ m of primary and secondary branches (Supplementary Fig. 4E). There were no significant differences in the spine density of BLA and LA pyramidal neurons between WT and

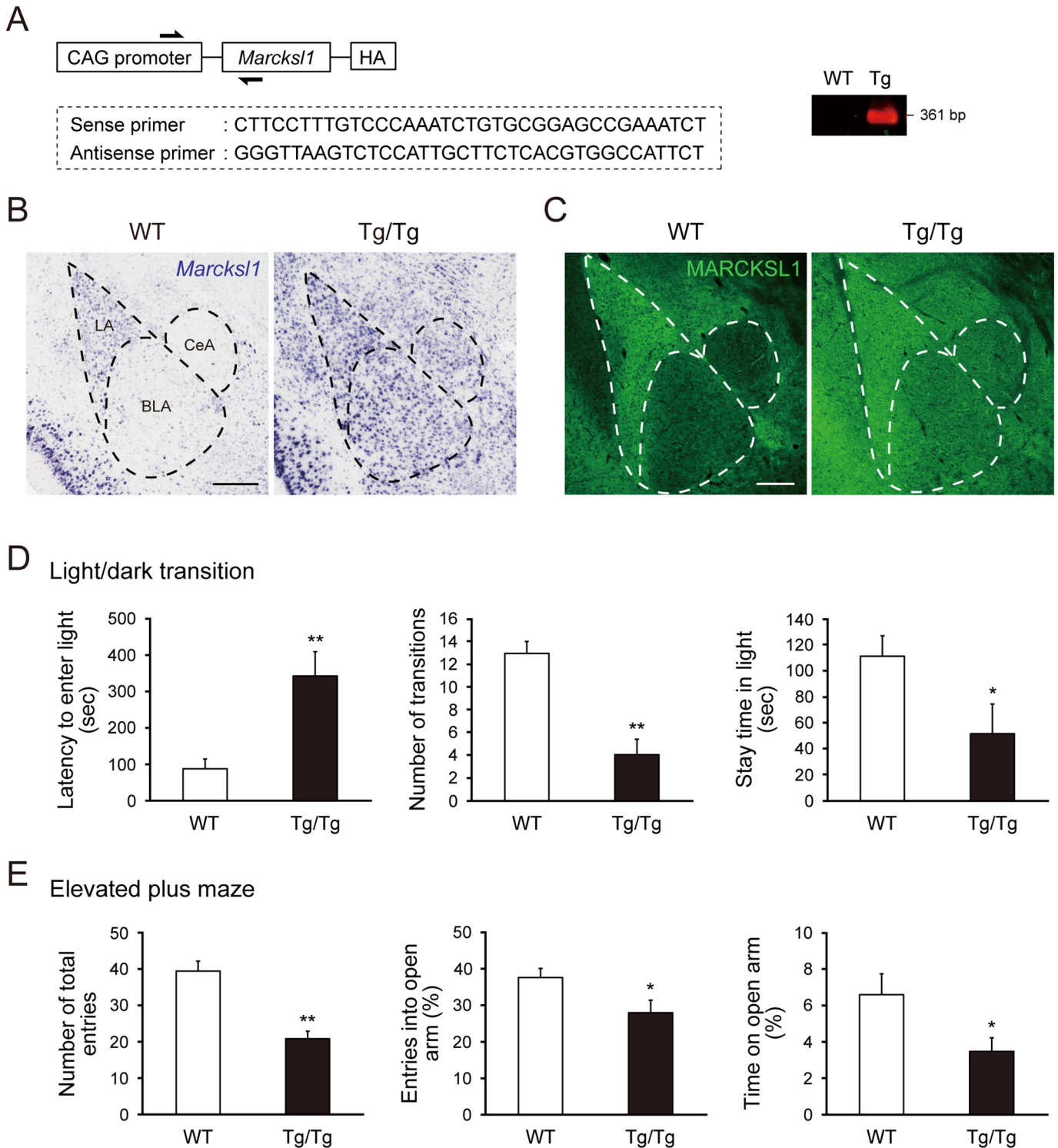


Fig. 2. Overexpression of MARCKSL1 induces anxiety-like behaviors. (A) Schema of the genomic organization of *Marcks11* inserted into a pCAGGS vector. The arrows represent the positions of genotyping primers. The dotted line box indicates the primer sequences. The right red bands are the result of genotyping. (B) In situ hybridization for *Marcks11* mRNA (blue) in the amygdala (three areas surrounded by the dotted lines) in WT and Tg/Tg mice. Scale bar, 200 μ m. (C) MARCKSL1 (green) staining in the amygdala (three areas surrounded by the dotted lines) in WT and Tg/Tg mice. Scale bar, 200 μ m. (D and E) Light/dark transition test scores and elevated plus maze test scores in WT and Tg/Tg mice (each group, $n = 11$). Data are presented as means \pm SEM. * $p < 0.05$, ** $p < 0.01$ (Student's *t*-test).

Tg/Tg mice (Supplementary Fig. 4F–I). These findings suggest that MARCKSL1 may play a functional role in anxiety-like behaviors by controlling dendritic spine formation in amygdala spiny neurons.

We next examined neural activity in amygdala neurons by measuring c-Fos expression under basal conditions in Tg/Tg mice (Supplementary

Fig. 5A). The number of c-Fos-positive cells in the CeA was significantly decreased in Tg/Tg mice (Supplementary Fig. 5B). In contrast, there were no significant changes in c-Fos-positive cell numbers in either the BLA or LA (Supplementary Fig. 5C and D). These results suggest that the CeA circuitry is hypoactive in Tg/Tg mice.

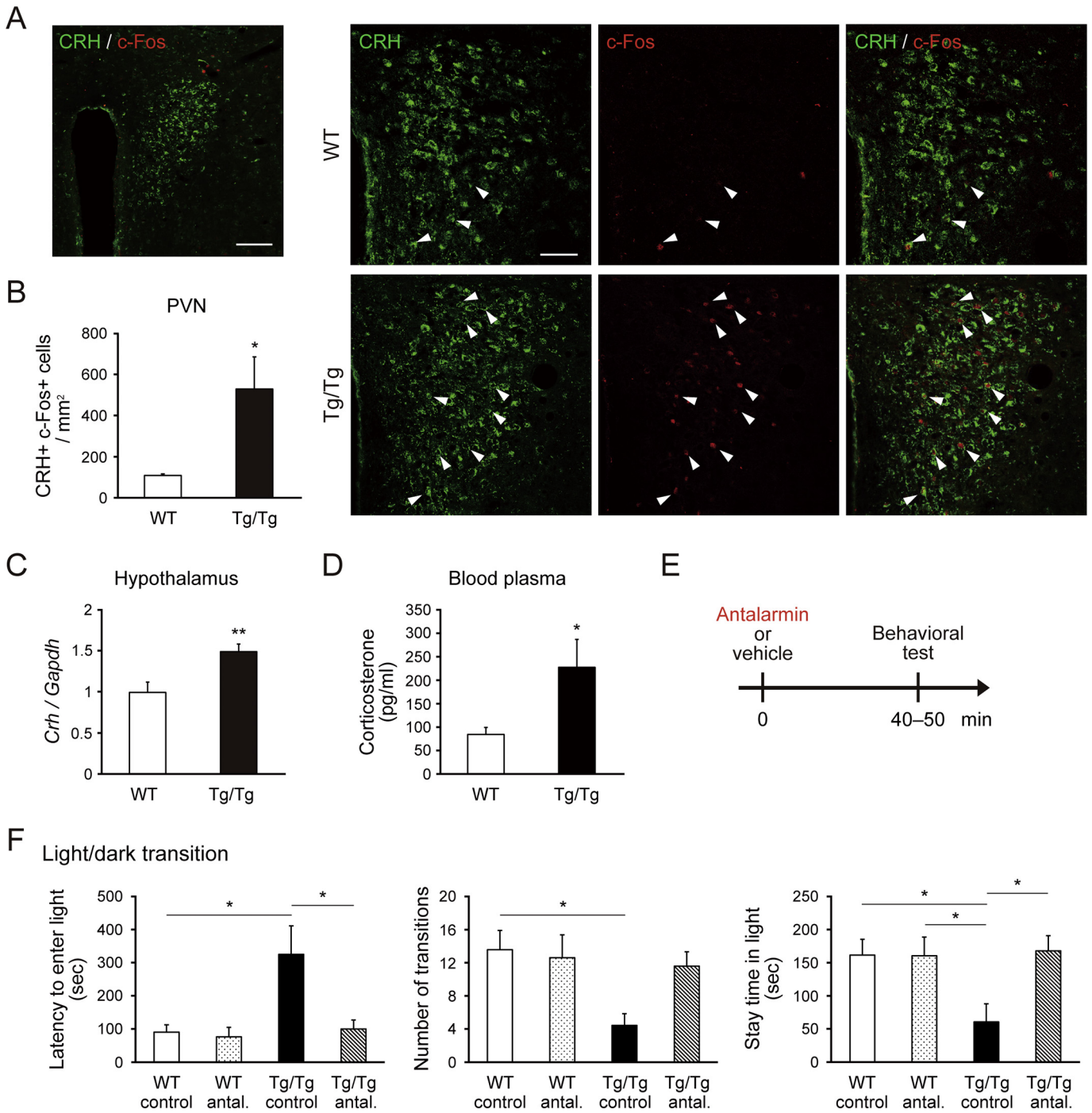


Fig. 3. CRH antagonism reduces anxiety-like behavior in MARCKSL1 Tg mice. (A) Coronal brain sections showing immunohistochemical staining of CRH (green) and c-Fos (red) in the PVN. Scale bar: *left* (low magnification), 100 μ m; *right* (high magnification), 50 μ m. (B) The number of CRH and c-Fos double-positive cells in the PVN of WT and Tg/Tg mice (each group, n = 4). Data are presented as means \pm SEM. *p < 0.05 (Student's *t*-test). (C) Expression of *Crh* mRNA in the hypothalamus was measured with real-time PCR (WT mice, n = 6; Tg/Tg mice, n = 7). Data are presented as means \pm SEM. **p < 0.01 (Student's *t*-test). (D) Blood plasma corticosterone concentration was quantified with ELISA (each group, n = 3). Data are presented as means \pm SEM. *p < 0.05 (Student's *t*-test). (E) Antalarmin (CRHR1 antagonist) or 4% DMSO (vehicle control) was infused into the peritoneal cavity 40–50 min prior to light/dark transition test. (F) Light/dark transition test scores by antalarmin injection (WT control, n = 7; WT antalarmin, n = 5; Tg/Tg control, n = 7; Tg/Tg antalarmin, n = 5). Data are presented as means \pm SEM. *p < 0.05 (one-way ANOVA followed by Tukey-Kramer test).

3.5. Downregulation of MARCKSL1 in the CeA Ameliorates Anxiety-Like Behaviors

To determine whether MARCKSL1 upregulation in the amygdala was functionally involved in anxiety-like behaviors of Tg/Tg mice, we used RNA interference to perform a knockdown of MARCKSL1 in the CeA of Tg/Tg mice. We targeted CeA rather than BLA neurons, since

the CeA contains the central inhibitory circuit of the amygdala, and CeA output inhibitory neurons target the hypothalamus via the bed nucleus of the stria terminalis (BNST) (Cassell et al., 1986; LeDoux et al., 1988).

First, we determined the most effective *Marcks1* siRNA to knock-down expression in vitro using western blotting (Supplementary Fig. 6A and B). The effect was verified in vivo using immunohistochemistry

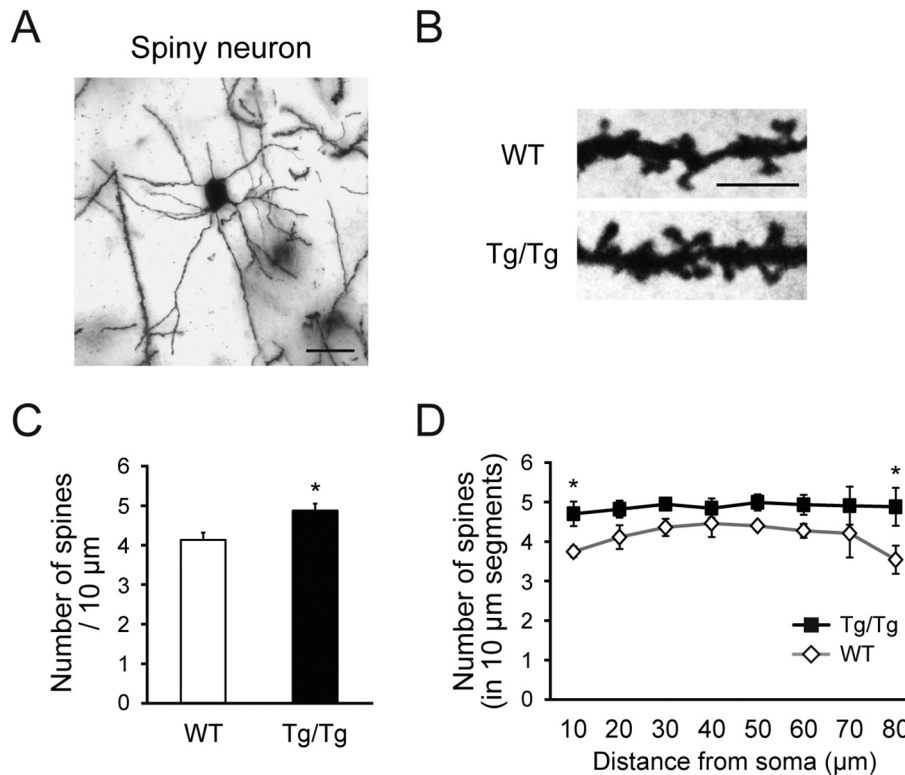


Fig. 4. Spine numbers of CeA spiny neurons are increased in MARCKSL1 Tg mice. (A) Low magnification photomicrograph of representative Golgi-stained spiny neurons in the CeA. Scale bar, 50 μm . (B) High magnification of representative Golgi-stained dendritic segments of spiny neurons in the CeA. Scale bar, 10 μm . Spines were counted, starting from the origin of a branch and advancing in 10 μm steps. (C and D) Spine numbers per 10 μm length of dendrite from cell soma of spiny neuron in the CeA (WT mice, $n = 3$; Tg/Tg mice, $n = 4$). Data are presented as means \pm SEM. * $p < 0.05$ (Student's t -test).

(Supplementary Fig. 6C). The selected *Marcks11* siRNA #2 was then infused into the CeA of Tg/Tg mice with i-Fect siRNA transfection reagents for 5 days prior to behavioral testing (Fig. 5A and B). In vivo *Marcks11* siRNA transfection effectively decreased *Marcks11* mRNA expression in the CeA for at least 10 days (Fig. 5C).

In the light/dark transition test, latency to enter the light box in *Marcks11* siRNA-transfected Tg/Tg (Tg/Tg + *Marcks11* siRNA) mice recovered to the same level as scramble control siRNA-transfected WT (WT + control siRNA) mice (Fig. 5D). The number of transitions and the time spent in the light box by Tg/Tg + *Marcks11* siRNA mice recovered to similar levels as those observed in WT + control siRNA mice (Fig. 5D). In the elevated plus maze, Tg/Tg + *Marcks11* siRNA mice tended to show a similar number of total arm entries, duration of time spent in open arms, and number of entries into open arms, compared with WT + control siRNA mice (Fig. 5E). These results demonstrate that downregulation of MARCKSL1 in the CeA reduced anxiety-like behaviors in Tg/Tg mice.

3.6. MARCKSL1 Downregulation in the CeA of MARCKSL1 Tg Mice Reduces Activity of CRH Neurons in the PVN

The number of CRH and c-Fos double-positive cells in the PVN of Tg/Tg + *Marcks11* siRNA mice was reduced compared with that in the PVN of WT + control siRNA mice (Fig. 6A and B). Furthermore, *Crh* mRNA levels (Fig. 6C) and corticosterone concentration (Fig. 6D) was decreased in Tg/Tg + *Marcks11* siRNA mice. Hence, downregulation of MARCKSL1 in the CeA of Tg/Tg mice reduced hyperactivity of CRH neurons in the PVN and subsequently reduced corticosterone levels in the blood plasma. To summarize, MARCKSL1 expression in the CeA appears to play a critical role in anxiety-like behaviors.

3.7. Brain Injury Increases MARCKSL1 Expression and Induces Anxiety-Like Behaviors

We previously investigated the molecular mechanism that mediates neurological deficits and recovery of motor functions in a traumatic brain injury model (Tanaka et al., 2013; Ueno et al., 2012). In this study, we were subsequently interested in the neuronal mechanism that may lead to altered emotional behaviors following injury. Indeed, a previous report indicated that approximately 53% of patients with traumatic brain injury experience major depressive disorder, and this is associated with an increased risk of developing an anxiety disorder (Bombardier et al., 2010).

We first examined whether anxiety-like behaviors were observed following brain injury in our model. The injury was induced in the left motor cortex (Supplementary Fig. 7A and B). In the light/dark transition test, the injured mice showed a significantly increased latency to enter the light box, and made fewer transitions between dark and light boxes (Fig. 7A). In the elevated plus maze test, mice with injury showed fewer total entries into the maze arms, and remained for shorter durations in the open arms (Fig. 7B). In our model, *Crh* mRNA levels in the hypothalamus was significantly increased 14 days post injury (Fig. 7C), and plasma corticosterone levels were also upregulated (Fig. 7D).

To investigate the role of MARCKSL1 in this process, we examined whether brain injury affected MARCKSL1 expression in brain regions associated with emotional processing. Real-time PCR analysis revealed that *Marcks11* mRNA was increased in the PFC and amygdala following injury, but not in the hypothalamus or hippocampus (Fig. 7E and Supplementary Fig. 7C). Consistent with mRNA expression, we also observed that MARCKSL1 immunoreactivity was increased in the amygdala following brain injury, most markedly in the CeA (Fig. 7F). These

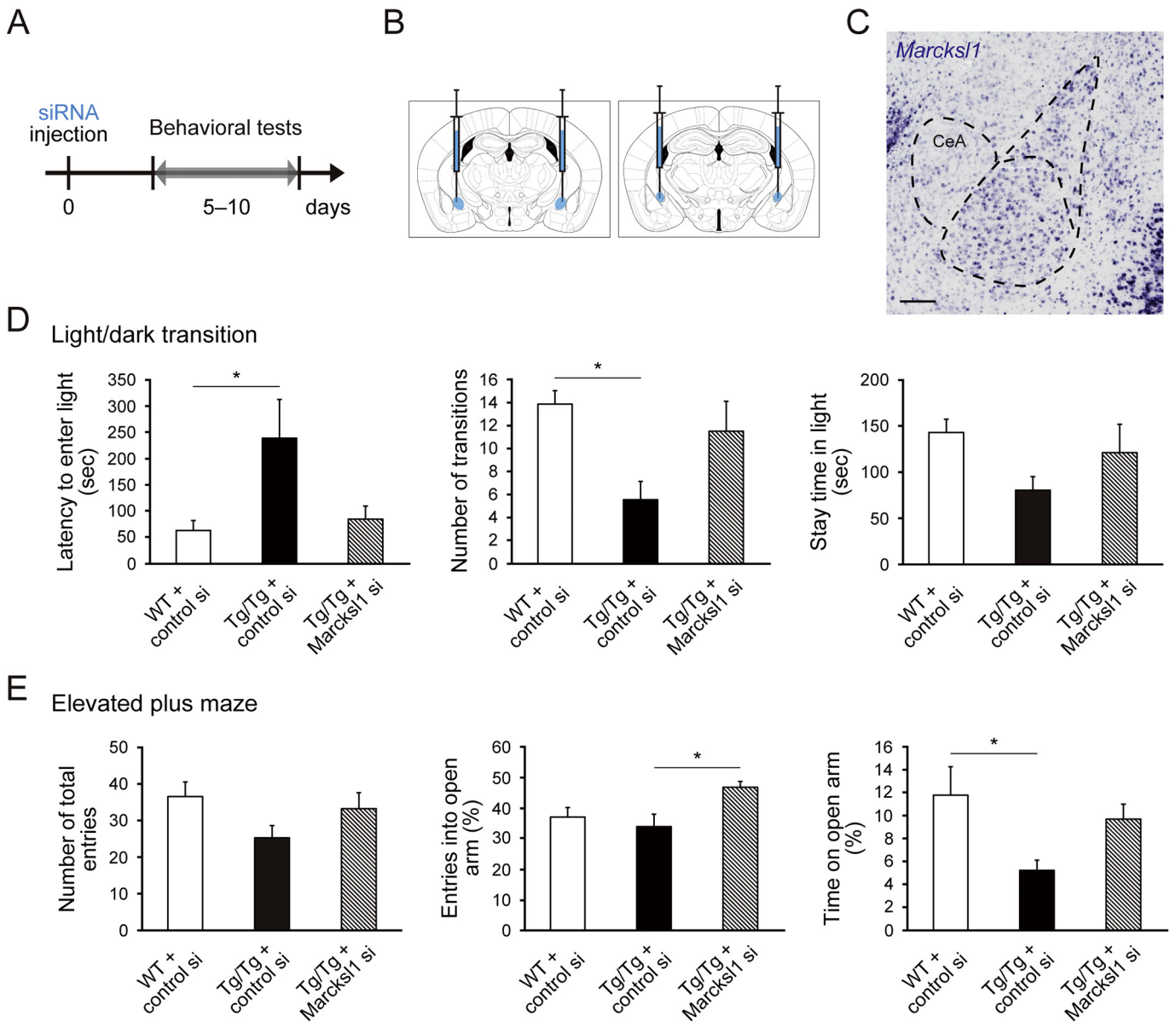


Fig. 5. Knockdown of MARCKSL1 ameliorates anxiety-like behavior in MARCKSL1 Tg mice. (A and B) For the in vivo experiment, siRNA (blue) was injected into the CeA (total 4 sites) with i-Fect siRNA transfection reagents 5 days prior to behavioral tests. (C) In situ hybridization for *Marcks1* mRNA (blue) in the amygdala after injection of *Marcks1* siRNA into the CeA of Tg/Tg mice. Scale bar, 200 μ m. (D and E) Light/dark transition test and elevated plus maze performance in MARCKSL1 knockdown mice (WT + control siRNA, $n = 7$; Tg/Tg + control siRNA, $n = 7$; Tg/Tg + *Marcks1* siRNA, $n = 8$). Data are presented as means \pm SEM. * $p < 0.05$ (one-way ANOVA followed by Tukey-Kramer test).

results suggest that increased MARCKSL1 expression in the amygdala implies alteration of local circuitry, implicating the generation of emotional behaviors.

4. Discussion

Our results demonstrate that MARCKSL1 upregulation in the CeA promotes anxiety-like behaviors, both in MARCKSL1 Tg and brain injury model mice. Here, the data suggest that MARCKSL1 plays a role in increasing spine formation in the local amygdala circuit that affects the output pathway to the hypothalamus, which increases the activity of the HPA axis and results in the induction of anxiety-like behaviors. Thus, we propose that regulation of MARCKSL1 in the amygdala plays a pivotal role in anxiety disorders.

In concordance with anxiety-like behaviors, we found that MARCKSL1 Tg mice showed signs of hyperactivity in the HPA axis. The

mice exhibited CRH upregulation, CRH neuron activation in the PVN, and consistent upregulation of plasma corticosterone levels. In addition, CRHR1 suppression effectively attenuated anxiety-like behaviors. Indeed, many previous studies have demonstrated that corticosterone is the final product of the HPA axis that triggers stress responses (Herman and Tasker, 2016; Matys et al., 2004; Vale et al., 1981). Furthermore, the HPA axis response is dysregulated via continuous hyperactivity of CRH neurons in the PVN of patients with major depressive and anxiety disorders (de Kloet et al., 2005; Dedic et al., 2012). Altogether, our data suggest that anxiety-like behaviors in MARCKSL1 Tg mice are evoked by hyperactivation of the HPA axis via a CRHR1-dependent mechanism.

We next observed two abnormalities in amygdala neurons of MARCKSL1 Tg mice. First, spiny neurons in the CeA and BLA of Tg mice showed significantly increased numbers of dendritic spines. Second, c-Fos-positive, active neurons in the CeA were significantly decreased in

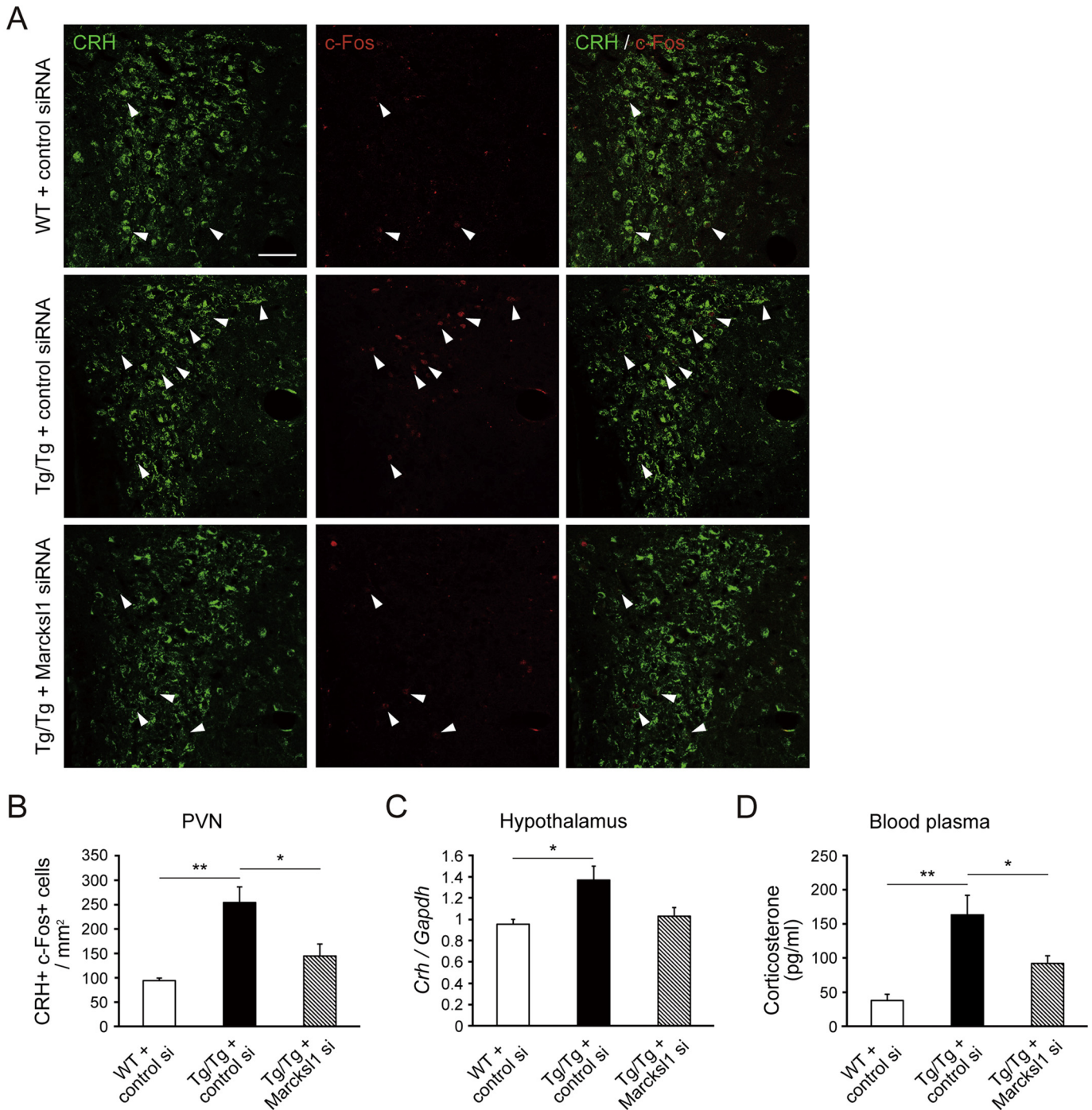


Fig. 6. MARCKSL1 inhibition in the CeA reduces the activity of CRH neurons in the PVN. (A) Immunohistochemical staining of CRH and c-Fos in the PVN of control or *Marcks11* siRNA-injected mice. Scale bar, 50 μ m. (B) The number of CRH and c-Fos double-positive cells in the PVN of control or *Marcks11* siRNA-injected WT and Tg/Tg mice (each group, $n = 4$). Data are presented as means \pm SEM. * $p < 0.05$, ** $p < 0.01$ (one-way ANOVA followed by Tukey-Kramer test). (C) Relative expression of *Crh* mRNA in the hypothalamus examined with real-time PCR (each group, $n = 3$). * $p < 0.05$ (one-way ANOVA followed by Tukey-Kramer test). (D) Corticosterone concentration in the blood, quantified with ELISA (WT + control siRNA, $n = 3$; Tg/Tg + control siRNA, $n = 3$; Tg/Tg + *Marcks11* siRNA, $n = 4$). * $p < 0.05$, ** $p < 0.01$ (one-way ANOVA followed by Tukey-Kramer test).

Tg mice. The CeA consists of inhibitory spiny neurons that target the hypothalamus via the BNST (Cassell et al., 1986; LeDoux et al., 1988). Because elevated expression of MARCKSL1 affected the spines in and activity of the CeA, and subsequently led to HPA axis activation, anxiety-like behaviors in Tg mice might be evoked through dysregulated amygdala output via the hypothalamic pathway. Indeed, our RNA interfering experiments support this neuronal mechanism. Specific knock-down of MARCKSL1 expression in the CeA suppressed the activity of

CRH neurons in the PVN and rescued anxiety-like behaviors in MARCKSL1 Tg mice. Thus, MARCKSL1 in CeA may be one of the key molecules that regulate activity of CRH neurons in the PVN and thus controls states of anxiety.

The precise mechanism by which MARCKSL1 controls spine numbers is still unclear. It is well known that the actin cytoskeleton is essential for the formation and stabilization of filopodia, which is an immature dendritic spine, and for spine morphology. PKC and Jun N-terminal protein

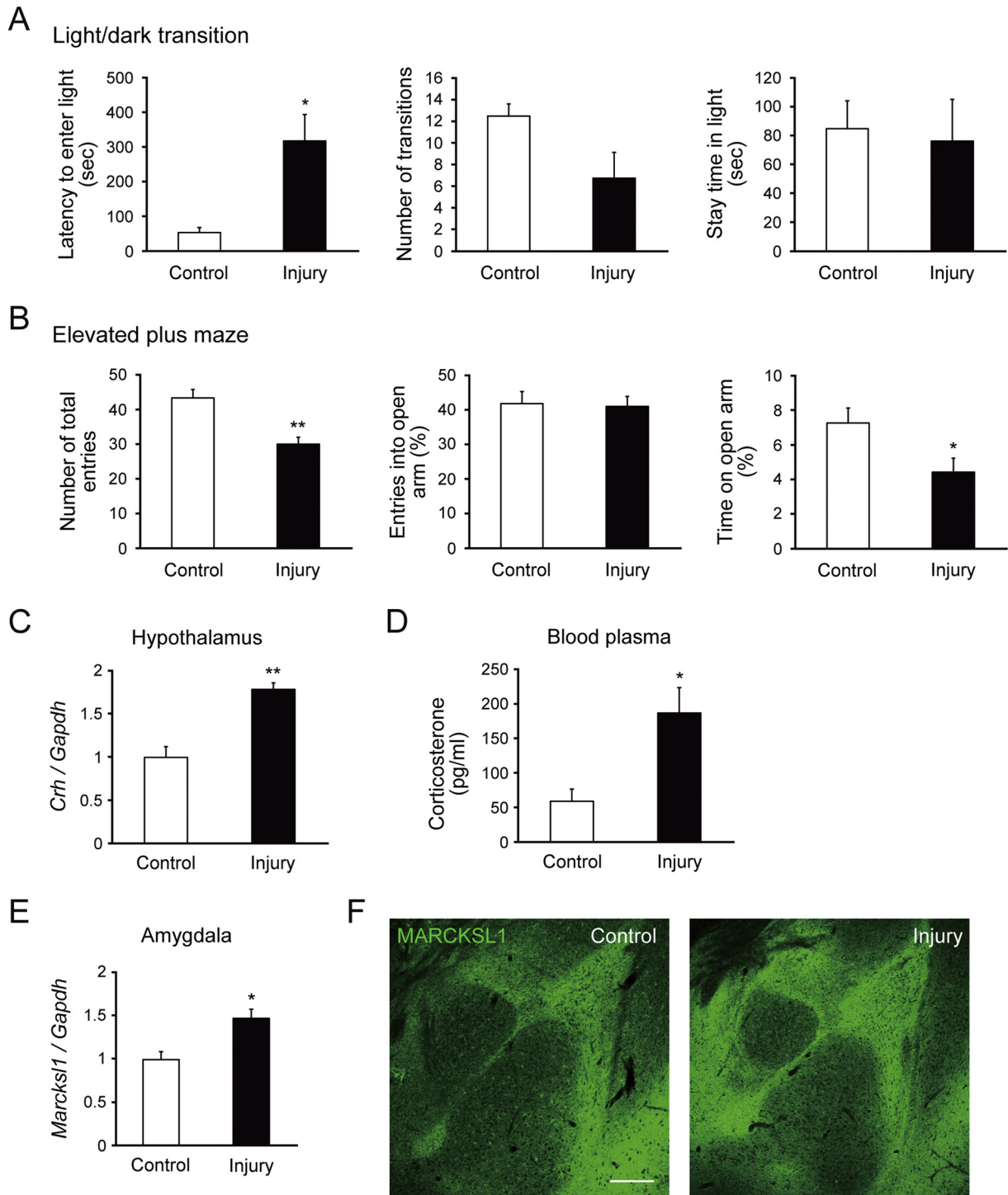


Fig. 7. Brain injury induces anxiety-like behaviors and MARCKSL1 expression in the amygdala. (A and B) Light/dark transition test and elevated plus maze performance in WT mice after brain injury (control mice, $n = 6$; injury mice, $n = 8$). Data are presented as means \pm SEM. * $p < 0.05$, ** $p < 0.01$ (Student's t -test). (C) Relative expression of *Crh* mRNA in the hypothalamus of mice with brain injury examined with real-time PCR (control mice, $n = 6$; injury mice, $n = 8$). Data are presented as means \pm SEM. ** $p < 0.01$ (Student's t -test). (D) Corticosterone concentration in blood plasma was quantified with ELISA (control mice, $n = 5$; injury mice, $n = 7$). Data are presented as means \pm SEM. * $p < 0.05$ (Student's t -test). (E) Relative expression of *Marcks11* mRNA in the amygdala of mice with brain injury, examined with real-time PCR (control mice, $n = 4$; injury mice, $n = 8$). Data are presented as means \pm SEM. * $p < 0.05$ (Student's t -test). (F) MARCKSL1 (green) staining in the amygdala of control and 14-days post-injury mice. Scale bar, 200 μ m.

kinase (JNK) are known to regulate actin stability through phosphorylation of MARCKSL1 (Arbuzova et al., 2002; Björkblom et al., 2012; Sundaram et al., 2004). Neurons expressing MARCKSL1 or MARCKSL1 phosphorylated by JNK promote neuronal dynamics such as increasing filopodia numbers along the dendrites by bundling F-actin (Björkblom

et al., 2012). In this view, MARCKSL1 would be an important intrinsic factor that regulates dendritic spine formation. Interestingly, previous studies have indicated that JNK1 regulates dendritic architecture and synaptic plasticity in neurons (Chang et al., 2003; Coffey, 2014; Komulainen et al., 2014), and that inhibition or knockout of JNK1 induces

an anxiolytic effect in mice (Mohammad et al., 2018; Reinecke et al., 2013). Given that JNK1 is a dominant regulator of MARCKSL1 actin bundling, it is highly relevant that JNK1 knockout mice show a low anxiety phenotype and MARCKSL1 overexpression increased anxiety. Further investigation is required to understand the mechanism by which MARCKSL1 overexpression increases the dendritic spines of spiny neurons. We also do not know whether MARCKSL1 controls dendritic spine formation in other neuronal subtypes and brain regions. It is possible that MARCKSL1-mediated structural changes in other neuronal systems control brain function.

In conclusion, our study demonstrates the potential involvement of MARCKSL1 in the regulation of anxiety-like behaviors in mouse models. Although MARCKSL1 overexpression induced clear anxiety-like behaviors, the neuronal mechanisms underlying how the increased spines in amygdala spiny neurons decrease activity in the CeA and subsequently activate the HPA axis remain unclear. In addition, the physiological roles of MARCKSL1 on anxiety behaviors in various living situations have not been investigated in this study. Hence, it will be important to conduct further multidirectional analyses to understand the underlying mechanisms and neural substrates of anxiety. These analyses will require the use of multiple experimental models, such as stress-exposure and brain injury models, in both inbred mice strains and genetically engineered mice, ranging from early developmental stages to adulthood. Elucidating the functional roles of MARCKSL1 in the CeA and BLA should be one of the primary goals of future research.

Original data of this study are available at Mendeley Data (<http://dx.doi.org/10.17632/xgrdgpj82.1>).

Funding Sources

This work was supported in part by the Japan Society for the Promotion of Science, a Grant-in-Aid for Scientific Research (C) (KAKENHI; grant 25430079), and The Osaka Medical Research Foundation for Intractable Diseases.

Conflicts of Interest

The authors declare no competing financial interests.

Author Contributions

T.T. conceived the project, designed and performed the experiments, analyzed the data, and wrote the manuscript. S.S. performed in situ hybridizations and in vitro cell culture experiments. Y.F. and M.I. did generation of transgenic mice and technical assistance. M.U. searched literature and wrote the manuscript. S.M. supervised this project.

Acknowledgements

We are grateful to Drs. T. Ishikawa and T. Katayama (Osaka University) for providing reagents and vectors. We thank NPO Biotechnology Research and Development for technical assistance. We thank Dr. Y. Koyama (Osaka University) for their valuable comments and suggestions. We would like to thank N. Mizuguchi and A. Tatemizo (Center for Animal Experiment, Kindai University) for conducting the transgenic mice experiments. We would like to thank Editage (www.editage.jp) for English language editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.03.018>.

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