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Research Paper

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

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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.

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 *Marcksl1* 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 *Xho*I and *Eco*RI site of a pCAGGS vector (Addgene) by introducing *Marcksl1* containing a HA tag (Fig. 2A). The amplified DNA plasmid was treated with *Bam*HI, *Sca*I, and *Sal*I to prepare a 3.1 kb fragment, which was microinjected into the pronucleus of fertilized B6D2F1 mouse eggs. Male and female mice expressing the *Marcksl1* transgene were identified with Southern blotting. Heterozygous offspring were mated to produce homozygous mutant animals. Genotypes at the pCAGGS-*Marcksl1* 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′-CTTCCTTTGTCCCAAATCTGTGCGGAGCCGAAATCT-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 *Marcksl1* siRNA (Sigma-Aldrich) containing the following sequences: sense (5′-CAUCAGCCAUUUGGUCUUATT-3′) and antisense (5′-UAAGACCAAAUGGCUGAUGTT-3′). *Marcksl1* 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 *Marcksl1* 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

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