Deletion of the kinase domain from death-associated protein kinase enhances spatial memory in mice

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Received October 18, 2005; Accepted December 5, 2005

Abstract. Death-associated protein kinase (DAPK) is a Ca^{2+}/calmodulin-dependent serine/threonine kinase that is thought to mediate apoptosis. DAPK is highly expressed in hippocampal neurons which are essential elements for memory formation. To examine if DAPK is implicated in spatial learning and memory, both wild-type and DAPK-mutant mice were subjected to Morris water maze tests. DAPK-mutant mice were generated by deleting 74 amino acids from the catalytic kinase domain of DAPK, and were used to investigate roles of the DAPK kinase domain in regulating spatial memory. Both mutant and wild-type mice were able to learn the water maze tasks to locate a hidden escape platform. In the first probe test, mutant mice showed a more precise memory for platform position compared to wild-type mice. In the reversal training in which the platform was located opposite from the original position, DAPK-mutant mice exhibited superior spatial learning compared to wild-type mice. DAPK-mutant mice also showed a more precise memory than their wild-type littermates in the probe trial of reversal test. Thus, the present results revealed crucial implications of DAPK in regulating spatial memory in mice.

Introduction

Death-associated protein kinase (DAPK) is a Ca^{2+}/calmodulin (CaM)-regulated serine/threonine kinase, originally identified as an apoptotic mediator using a functional gene cloning method (1-3). DAPK is known to modulate apoptosis induced by several cytokines, Fas, ceramide, and cellular detachment from the extracellular matrix (4-7). Studies using knockout mice revealed crucial roles of DAPK in apoptotic cell death induced *in vitro* and *in vivo* (5,8-11). Thus, many studies, including ours, showed that DAPK was linked to apoptosis, by disclosing its pro-apoptotic role. However, several reports showed that DAPK also exhibited an anti-apoptotic role in certain growth conditions (12,13). This suggested that DAPK might be able to change its manner of functioning to suit the cellular context (2,14).

DAPK is highly expressed in hippocampal neurons which are essential elements for memory storage (15-17). It can interact with various proteins through its protein interaction domains residing in a 160-kDa multidomain protein (2,17-21). Binding of calmodulin (CaM) to DAPK regulates the kinase activity of DAPK (2,18). CaM displays an important role as a signal integrator for synaptic plasticity and memory (22). Localization of DAPK to actin stress fiber induces disruptions of actin microfilaments and cytoskeleton organization to mediate morphological changes of cultured cells (23). Recent studies implicated actin and cytoskeleton in the morphological plasticity of synapses (24). DAPK can interact with syntaxin-1A, a key component of synaptic vesicle docking/fusion machinery (25). Through its death domain, DAPK also interacts with the extracellular signal-regulated kinase (ERK) which has an essential role in synaptic plasticity (20). Thus, DAPK may exhibit certain functions in learning and memory through interactions with synaptic plasticity-related proteins. To identify if DAPK plays a role in memory processes, we subjected DAPK-mutant mice to Morris water maze tasks.

Materials and methods

DAPK-mutant mice. Mice expressing a mutant form of DAPK lacking a portion of the kinase domain (amino acids 22-95), were generated by gene targeting in E14.1 embryonic stem (ES) cells (8-11). Briefly, targeting vectors were designed to replace the exon encoding amino acids 22-95 of mature DAPK with a neomycin-resistance gene. Vectors were introduced into E14.1 ES cells using electroporation. Clones resistant to G418 and ganciclovir were screened by PCR, and confirmed by Southern blot analysis. Mutant ES cells were injected into

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Key words: death-associated protein kinase, knockout mouse, memory, water maze

blastocysts (C57BL/6), and transferred to uteri of pseudo-pregnant mice to generate chimeras. Chimeras were bred with C57BL/6 mice for germ-line transmission of the mutant allele. Pairs of the resultant heterozygous mice were then bred to obtain homozygous DAPK-mutant mice. The resulting mice were backcrossed with C57BL/6 mice. This study used F6 generation knockout mice and their wild-type littermates as controls. Mice were housed in the animal facilities of Wakayama Medical University. Our institutional Animal Ethics Review committee approved the experimental protocols.

Open-field test. Each mouse was placed in a circular open field area (80-cm diameter), and allowed to freely explore the environment for 20 min. Horizontal activities of 9 wild-type and 9 mutant mice were measured using a computer-assisted video tracking system (CompACT vas, Muromachi Kikai, Tokyo, Japan).

Water maze analysis. The spatial learning and memory of 9 male wild-type and 9 male mutant mice were tested using a Morris water maze consisting of a circular plastic pool (120-cm diameter, 25-cm deep). The pool was located in a rectangular room (220-cm wide x 260-cm long x 240-cm high) with a lot of visual cues. For swimming tracking, a small TV camera was fixed to the end of a metal rod extending over the pool. Mice were trained to locate a hidden escape platform over 14 daily two-trial sessions. For each task, each mouse was required to locate, and climb onto the hidden circular platform (11-cm diameter) submerged 1 cm below the surface of opaque water, at a temperature of 25±1˚C. The platform was located at the center of the quadrant of the pool, and its position was fixed throughout the hidden platform task. Mice were allowed to search for the platform for 60 sec, and time spent to reach the platform (latency) was recorded. During the probe trial on the 15th day, the platform was removed, and each mouse was allowed to search in the pool for 60 sec. Then, the platform was moved to the opposite position, and reversal learning was monitored for 14 additional days (2 trials per day), followed by second probe trials. Both quadrant search times and platform crossings were measured post-hoc from videotape recordings of probe trials.

Passive avoidance learning test. For the step-through type passive avoidance learning test, mice were trained to use an apparatus consisting of a small well-lit and larger dark compartment (16x11 cm base, 11-cm high), and a guillotine door separating the two compartments. The larger compartment was in darkness owing to its construction with black plexiglass and a removable cover. This compartment had a grid floor through which a constant current shock could be delivered. The smaller compartment was made of white plexiglass, and was illuminated with a tensor lamp. On the training day, each mouse was placed in the lit compartment, and was allowed to freely explore for 10 sec. The door to the dark compartment was then opened, and step-through latency was recorded as the time the mouse took to enter the dark compartment using all four paws. After the mouse stepped through the door, the door was closed, and 2 foot shocks (0.4 mA, 50 Hz, 2 sec) were delivered at 5-sec interval. The mouse was then removed from the apparatus, and returned to its home cage. The retention test was similarly performed 24 h later except that no shock was delivered. A maximum step-through latency of 300 sec was recorded in the test session.

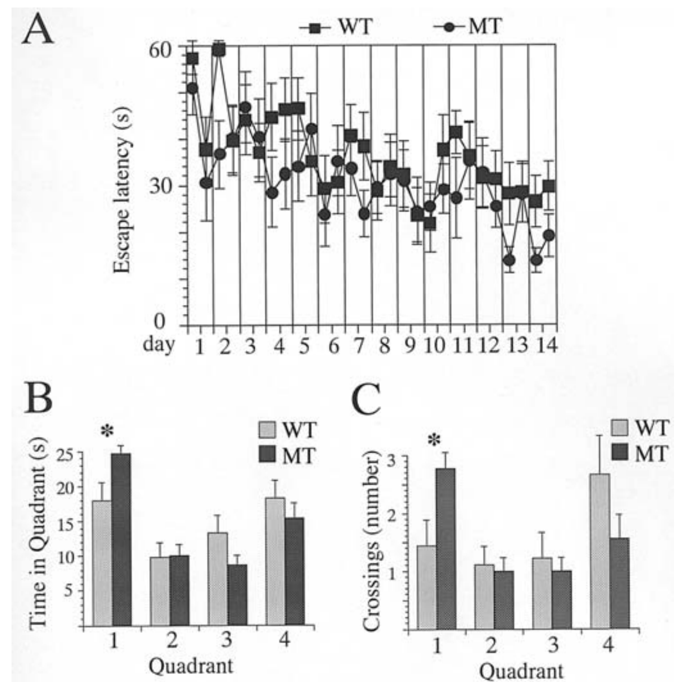

Figure 1. Enhanced spatial memory in DAPK-mutant mice. (A) Morris water maze training to find the hidden platform. For each trial, mean (± SEM) latency to reach the platform is plotted as a spatial learning function of the DAPK kinase domain (WT, wild-type; MT, mutant). (B and C) Transfer test performed one day after the 14-day training period. Values represent all four quadrants: 1, training quadrant; 2, adjacent left quadrant to the goal; 3, opposite quadrant to the goal; 4, adjacent right quadrant to the goal position. Mutant mice spent more time in the target quadrant (B), and displayed a more precise memory in crossing number for target platform position (C). *P<0.05, Dunnett's test.

Statistics. Statistical analyses were conducted using StatView (Abacus Concepts, Berkeley, CA). Unless specified otherwise, data were analyzed using an analysis of variance. A P-value <0.05 was regarded as statistically significant. Values were presented as means ± SEM.

Results

Enhanced spatial memory in DAPK-mutant mice. To examine if DAPK was implicated in spatial learning and memory, both wild-type and DAPK-mutant mice were subjected to Morris water maze tests (26). No difference was found in swim speed between wild-type and mutant mice (wild-type, 25.3±2.05 cm/sec; mutant, 26.7±3.17 cm/sec; Student's t-test, P>0.05). The ability of both groups to find the hidden platform improved significantly [F (27, 432) = 3.404, P<0.0001] during training trials (Fig. 1A), and there was no significant difference between the two groups [F (1, 16) = 2.142, P=0.1627]. However, in the first probe test after the 14 days of training, DAPK-mutant mice spent more time in the target quadrant than wild-type mice (P<0.05, Dunnett's test) (Fig. 1B), and crossed the platform sites more often (P<0.05, Dunnett's test) (Fig. 1C). Thus, DAPK-mutant mice showed a more precise memory for platform position compared to wild-type mice.

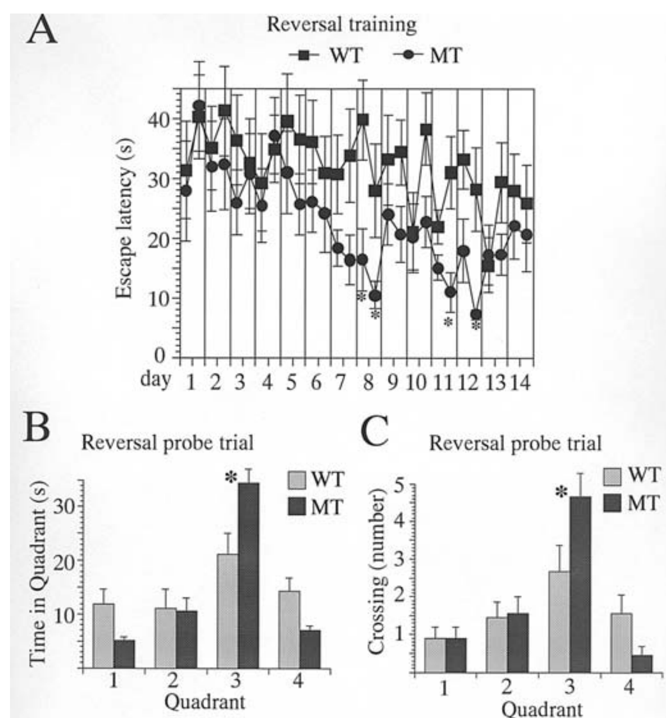

Figure 2. Enhanced spatial learning and memory in DAPK-mutant mice. (A) Reversal training to find the hidden platform located opposite to the original position. Mean (± SEM) escape latency per trial is plotted for 14-day training (WT, wild-type; MT, mutant). DAPK-mutant mice showed significantly shorter escape latencies than wild-type animals at 15th, 16th, 22nd and 25th trials. (B and C) A probe test was performed after the 14-day reversal training period. Values represent all four quadrants: 1, old training quadrant; 2, adjacent left quadrant to the old goal; 3, reversal training quadrant; 4, adjacent right quadrant to the old goal position. Mutant mice spent more time in the target quadrant (B), and displayed a more precise memory in crossing number for target platform position (C). *P<0.05, Dunnett's test.

To further confirm the superior spatial memory of DAPK-mutant mice, the target platform was moved to a new position opposite to the original site, and both groups of mice were trained again in the Morris water maze. The ability of both groups to find the hidden platform improved significantly [F (27, 432) = 2.424, P<0.05] during the training trials (Fig. 2A), and mutant mice exhibited shorter escape latencies than wild-type mice throughout the trials [F (1, 16) = 5.464, P<0.05]. A post-hoc Scheffe's test revealed significant differences between the two genotypes at the 15th (P<0.05), 16th (P<0.05), 22nd (P<0.05) and 24th trials (P<0.05). The results indicated an enhanced spatial learning of mutant mice in the second water maze task. In the second probe test, both wild-type and mutant mice spent more time in the target quadrant than in other quadrants [F (3, 48) = 17.375, P<0.0001], and crossed platform sites more often than the alternate sites [F (3, 48) = 13.557, P<0.05]. However, DAPK-mutant mice spent more time in the target quadrant than wild-type mice (P<0.05, Dunnett's test) (Fig. 2B), and crossed the platform sites more often (P<0.05, Dunnett's test) (Fig. 2C). Thus, DAPK-mutant mice also showed a more precise memory for platform position compared to wild-type mice in the second water maze test.

Normal behavior in other tests. In the open-field locomotor activity test performed before the Morris water maze task, main effect of group and interaction effects of time and group were not significant [F (1, 16) = 0.066; and F (3, 48) = 1.921, respectively]. However, main effect of time was significantly different [F (3, 48) = 43.87, P<0.0001]. Thus, all groups of animals moved normally, and habituated to experimental conditions within 20 min.

In the step-through type passive avoidance task following the Morris water maze test, step-through latency significantly increased after conditioning [F (2, 32) = 13.340, P<0.0001]. There was no significant difference in latency at 24 h after conditioning between the two groups [F (1, 16) = 0.365]. Thus, the contextual learning capacity in the passive avoidance test was similar in wild-type and DAPK-mutant mice.

Discussion

Our mouse behavioral analysis revealed a superior spatial memory of DAPK-mutant mice using Morris water maze tests. Thus, our findings suggested a crucial implication of DAPK in signal transduction pathways of hippocampal neurons during spatial learning and memory processes.

DAPK is thought to be a signal transducer, activated during the process of neuronal apoptosis in both cultured cells and animal disease models (5,6,17,19,27). Expression levels of DAPK increase in hippocampal neurons by either seizure or ischemia (17,19,28,29). DAPK is induced to associate with various kinds of apoptosis-related molecules in the hippocampus following seizures (17,19,28). Since DAPK is expressed in a neuron-specific manner in normal hippocampus (15-17), DAPK may physiologically act as a Ca^{2+}-dependent serine/threonine kinase in hippocampal neurons. Thus, the superior spatial memory of DAPK-mutant mice in our study was the first report suggesting a crucial implication of DAPK in the regulatory mechanisms of spatial learning and memory.

The superior spatial memory of DAPK-mutant mice was also supported by *in vitro* experimental data reported by other authors (20,25,32). DAPK can bind to syntaxin-1A, a key docking/fusion machinery component of synaptic vesicles, where DAPK phosphorylates syntaxin-1A in a Ca^{2+}-dependent manner (25). Thus, DAPK may control presynaptic neurotransmitter release, by regulating complex formation between syntaxin and Munc18, a necessary component for synaptic vesicle docking (25). Syntaxin expression was found to be upregulated in hippocampal circuits during spatial learning (30,31). Enhanced spatial learning and memory in DAPK-mutant mice might result from DAPK-mutant neurons responding in a hypersensitive manner to synaptic activity, due to dysregulation of Munc18-syntaxin complex formation. Seizure grade induced by kainic acid is significantly higher in DAPK-mutant mice compared to wild-type mice (Yukawa, unpublished data). This may imply enhanced presynaptic neurotransmitter release during hippocampal synaptic activity in DAPK-mutant mice. DAPK was recently shown to inactivate integrin function in cultured cells through an inside-out mechanism (32). Integrin signaling is an essential component for long-term potentiation, a synaptic plasticity form of learning and memory (33,34). Insufficient restriction of integrin signaling during long-term potentiation might cause enhanced spatial memory in DAPK-mutant mice. A study using cultured cells further demonstrated that DAPK

interacted with ERK and inhibited the nuclear translocation of ERK, leading to attenuation of ERK signaling (20). Activation of ERK signaling in hippocampal neurons is essential for long-term potentiation (35,36). Since mutant DAPK molecules may not efficiently restrict the nuclear translocation of ERK induced by synaptic activity, superior spatial memory may be observed in DAPK-mutant mice due to enhanced ERK signaling in hippocampal neurons during spatial learning.

In the present study, DAPK-mutant mice did not display enhanced memory in the passive avoidance learning test. Passive avoidance procedures measure the integrity of long-term synaptic processes in many regions including amygdala, striatum, and hippocampus (37). An absence of DAPK in amygdala and ventral caudate-putamen may account for the normal memory in DAPK-mutant mice in the passive avoidance learning test (17). Alternatively, the use of two electrical shocks in our study may be too strong to observe significant differences between the two genotypes. A passive avoidance test using a weaker shock stimulus might result in a superior retention of memory in DAPK-mutant mice. Since mutant DAPK molecules are presumably expressed in early mouse embryos, developmental abnormalities of the mutant brain may cause enhanced spatial memory in DAPK-mutant mice. Both intact brain architecture and normal open-field activity in mutant mice may lower such a possibility (Yukawa, unpublished data).

Future study directly testing the implications of DAPK in synaptic plasticity is required to demonstrate that DAPK plays a crucial role as a signal transducer in hippocampal neurons for retention of memory. Several ongoing investigations are developing therapeutic approaches to neuronal death by modulating DAPK activity (3,5,29,38-40). Our results suggest that DAPK is a good target for developing new therapies against memory disorders.

Acknowledgements

This study was partly supported by a grant to K.Y. and T.T. from Wakayama Medical University. We are grateful to H. Kikutani, K. Kubota, M. Kishino, and E. Yukawa for their encouragement and support.

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