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A mental retardation gene, motopsin/neurotrypsin/prss12, modulates hippocampal function and social interaction

Shinichi Mitsui[1,*], **Yoji Osako**[1], **Fumiaki Yokoi**[2], **Mai T. Dang**[3], **Kazunari Yuri**[1], **Yuqing Li**[2], and **Nozomi Yamaguchi**[4,5]

[1]Department of Neurobiology and Anatomy, Kochi Medical School, Okoh, Nankoku 783-8505, Japan

[2]Center for Neurodegeneration and Experimental Therapeutics, Department of Neurology, School of Medicine, University of Alabama at Birmingham, AL 35294

[3]Children's Hospital of Philadelphia, Philadelphia, PA 19104

[4]Department of Cell Biology, Research Institute for Geriatrics, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan.

Abstract

Motopsin is a mosaic serine protease secreted from neuronal cells in various brain regions including the hippocampus. The loss of motopsin function causes nonsyndromic mental retardation in humans and impairs long-term memory formation in *Drosophila*. To understand motopsin's function in the mammalian brain, motopsin knockout mice were generated. Motopsin knockout mice did not have significant deficit in memory formation, as was tested using in the Morris water maze, passive avoidance, and Y-maze tests. A social recognition test showed that the motopsin knockout mice had the ability to recognize two stimulator mice, suggesting normal social memory. In a social novelty test, motopsin knockout mice spent a longer time investigating a familiar mouse than wild-type mice did. In a resident-intruder test, motopsin knockout mice showed prolonged social interaction compared to wild-type mice. Consistent with the behavioral deficit, spine density was significantly decreased on apical dendrites, but not on basal dendrites, of hippocampal pyramidal neurons of motopsin knockout mice. In contrast, pyramidal neurons at the cingulate cortex showed normal spine density. Spatial learning and social interaction induced the phosphorylation of cAMP responsive element binding protein (CREB) in hippocampal neurons of wild-type mice, whereas the phosphorylation of CREB was markedly decreased in mutant mouse brains. Our results indicate that an extracellular protease, motopsin, preferentially affects social behaviors, and modulates the functions of hippocampal neurons.

Keywords

autism; CREB; developmental disorder; serine protease; spine

Introduction

Extracellular serine proteases, such as motopsin, are known to have various physiological and pathological roles in the central nervous system (Yoshida & Shiosaka, 1999; Shiosaka & Yoshida, 2000; Tomimatsu *et al*., 2002; Mitsui *et al*., 2007a). Motopsin is an extracellular

[*] To whom correspondence should be addressed. Tel: +81 88 880 2298; Fax: +81 88 880 2300; smitsui@kochi-u.ac.jp .
[5]Present address; Mam Flora Hospital, Okunoin 25-2, Okukaiinji, Nagaokakyo 617-0853, Japan.

serine protease that was independently and simultaneously identified by two groups (Gschwend *et al*., 1997; Yamamura *et al*.,1997). HUGO nomenclature approved the names prss12 and neurotrypsin as well as motopsin. Motopsin is secreted from neuronal cells in various brain regions such as the hippocampus, the cerebral cortex, and the cranial nerve nuclei (Gschwend *et al*., 1997; Iijima *et al*., 1999). Motopsin mRNA in the hippocampus and the cingulate cortex is expressed most abundantly during the first postnatal week, and then gradually its expression decreases, but continues into adult life (Iijima *et al*., 1999; Wolfer *et al*., 2001). During the perinatal period, the abundant expression of motopsin mRNA is observed in other regions as well, such as the olfactory system, cranial nerve nuclei, spinal cord, and the peripheral nervous system, suggesting that motopsin plays multiple roles in the developing nervous system.

Recently, a four-base pair deletion in exon 7 of the motopsin gene, which results in a premature stop codon, was identified in a family in which four out of eight children had nonsyndromic autosomal recessive mental retardation (MR) (Molinari *et al*., 2002). In *Drosophila*, the downregulation of a possible motopsin ortholog, tequila, impairs long-term memory formation, but not short-term memory (Didelot *et al*., 2006). The impaired long-term memory formation is restored by the recovery of tequila expression, suggesting that tequila is involved in neuronal plasticity rather than neuronal development in the insect brain. The involvement of motopsin in neuronal plasticity is speculated because motopsin protein is detected at the presynaptic bouton, somatic body, and proximal part of dendrites (Molinari *et al*., 2002; Mitsui *et al*., 2007b). In addition, facial nerve axotomy causes transient downregulation of motopsin mRNA, whose expression is restored as the function of the facial nerve is recovered (Numajiri *et al*., 2006). Recently, it has been reported that motopsin is secreted from hippocampal neurons in an activity dependent manner (Frischknecht *et al*., 2008) and that motopsin cleaves a proteoglycan, agrin, which is known to modulate spine formation (Reif *et al*., 2007).

To understand motopsin's role in cognitive function, motopsin knockout mice were generated and their behavior was analyzed in this study. Unexpectedly, spatial memory deficit was undetectable in motopsin knockout mice. Despite normal social memory, motopsin knockout mice showed prolonged interest in a familiar mouse and longer social interaction to an intruder mouse in a social memory test and a resident-intruder test, respectively. The spine density at the hippocampal CA1 region was significantly decreased in motopsin knockout mice. After spatial learning or social interaction, the phosphorylation of cAMP responsive element binding protein (CREB) in hippocampal neurons was stimulated in wild-type mice, but was significantly reduced in motopsin knockout mice. Thus, motopsin appears to modulate hippocampus-dependent behavior such as social behavior and hippocampal function in the mammalian brain.

Materials and methods

Experimental animals

All animal experiments were performed in accordance with Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan and approved by the Animal Experiment Committee at Kochi Medical School. Motopsin knockout mice were generated with a *PGKneo* cassette replacing exon 1 using methods presented in a previous report (Li *et al*., 1994) (supporting Fig. S1*A*), and backcrossed with C57BL/6 background mice for at least 10 generation (N10). Heterozygote motopsin mutant mice were bred to produce the wild-type and knockout mice used in this study. Mice were kept in a standard cage (18 × 27 × 13 cm) in a 12 h light/dark cycle with access to food and water *ad libitum*.

Behavioral analyses

Behavioral tests were performed using 14 to 20 week-old male littermates from four cohorts. Mice were housed individually at least one week before behavioral tests. The interval between tests was 3 to 7 days. The order of behavioral tests occurred in the following way: Cohort 1 - open field test, light/dark box test, elevated plus-maze, beam walking, footprinting test, olfactory test; cohort 2 - open field test, exploration test, Y-maze test, sociability and social novelty tests, elevated plus maze, resident-intruder test; cohort 3 - passive avoidance test, Morris water maze test; cohort 4 - social recognition test.

Motor behavior tests

The beam-walking test was performed with some modifications according to previously published methods (Dang *et al*., 2005). This test evaluates the fine motor coordination ability of mice as they are challenged to walk across a suspended narrow beam (n=19 for wild-type mice, n=18 for knockout mice). Animals were trained to traverse a medium round beam (17 mm diameter) in three consecutive trials each day for four days. After training was completed, the experiment commenced with recordings of beam transversal time and number of hind paw slips for each of the two trials per beam. In the test session, animals were made to cross a small round beam (10 mm in diameter) and a small square (5 mm) beam.

The footprint test is an analysis of the animal's gait. A runway with a dark goal box at the end was lined with a sheet of white paper. Fore- and hind paws of mice were painted with water-soluble non-toxic paint of different colors. Mice were allowed to walk across the runway and into the goal box. One set of prints was collected for each animal after it walked continuously across the runway. Of each set, the four center pairs of hind- and fore- paw prints were analyzed for stride length, base length, and distance of overlap of the paws on each side.

Morris water-maze test

A hidden version of the Morris water-maze test was conducted in a circular pool of 1.2 m in diameter using male littermates (n = 14 for wild-type mice, n = 14 for knockout mice) according to a previous report (Chan *et al*., 2003). The pool was filled with water made opaque by adding a white non-toxic powder (titanium (IV) oxide). A platform (10 cm in diameter) was submerged 1 cm below water level. The mice were given four training sessions per day at 60-min intervals for five consecutive days. The latency to the escape platform was measured. Twenty-four hours after the last training session, a probe trial was performed. The mouse was placed in the pool without the escape platform and allowed to swim for 60 s. All of the behaviors were videotaped through a CCD camera in the ceiling board. The swimming path length was analyzed using an Ethovision system (Noldus Information Technology bv, Wageningen, The Netherlands). To assess CREB phosphorylation in the hippocampus, on the day after the probe test, mice (n=5 for each genotype) were given two additional training sessions. The mice were perfused transcardially with 4% paraformaldehyde and brains were prepared 90 min after the last training.

Passive avoidance test

Passive avoidance test was performed using male naïve littermates (n=15 for wild-type mice, n = 13 for knockout mice) according to a standard procedure (King *et al*., 2003). On day 1, a mouse was placed in the bright chamber of the passive avoidance apparatus (Muromachi Kikai Co., Ltd., Tokyo, Japan) and allowed to move freely between the light (170 lux) and dark (below 1 lux) chambers for 5 min. On day 2, the mouse was placed in the

bright chamber. To confirm the habituation, latency to enter the dark chamber was measured (WT, 5.59 ± 1.29 sec; KO, 9.05 ± 4.28 sec, $t_{26} = -0.824$, $P = 0.417$). After the entry to the dark chamber, the door between compartments was closed, and an electric shock (0.25 mA, 2 sec, 50 Hz) was delivered through the grid floor. The mouse was returned to the home cage. On day 3, the mouse was again placed in the bright chamber, and the latency to enter the dark chamber was measured with a cutoff at 300 sec. Preliminarily, it was confirmed that WT and motopsin knockout mice had similar foot-shock sensitivity by measuring the latency to enter the dark box 24 hr after giving 0.25 mA or 0.16 mA of an electric shock on day 2 (Fig. S4A in Supporting information). To assess CREB phosphorylation in the hippocampus, brains were prepared 90 min after the test on day 3 as described above.

Y-maze test

The Y-maze test was performed using male littermates ($n = 8$ for wild-type mice, $n = 10$ for knockout mice) following a previously published protocol (Huang *et al*., 2006). The apparatus was made of stainless steel; each arm was 40 cm long and 4 cm wide at the bottom and 13 cm at the top surrounded by a 12 cm high wall. The floor of the maze was covered with white rubber, and each wall was covered with a different colored sheet (gray, yellow, blue). The mouse was placed in the center of the apparatus and allowed to explore for 7 min in dim light. Spontaneous alternation was assessed visually by scoring the pattern of entries into each arm, and was defined as successive entries into each of the three arms. Percentage of alternation was calculated as the ratio of actual alternations to possible alternations (= the number of total arm entries minus 2) multiplied by 100.

Social recognition test

Because male mice showed sexual behavior to an ovariectomized stimulator mouse, the social recognition test was performed using female littermates ($n = 13$ for wild-type mice, $n = 14$ for knockout mice) under bright conditions (130 lx) as described elsewhere (Ferguson *et al*., 2000). The stimulator mouse was presented to the subject for 1 min in the home-cage. The same stimulator mouse was subsequently presented 4 times at 10-min intervals. In the fifth trial, a new stimulator mouse was presented for 1 min. The time spent sniffing the new stimulator mouse was measured.

Social behavior test

The social behavior test was performed in dim light (< 10 lux) using male littermates ($n = 9$ for wild-type mice, $n = 10$ for knockout mice) following the procedure described by Moy *et al*. (2007). The test mice were habituated to a test chamber (30 × 45 × 25 cm) containing two small stainless-steel wire cages (11 cm in diameter, 15 cm high) for 10 min. An unfamiliar mouse (stranger 1, wild-type male) was placed into one of the two wire cages, and the subject was allowed to explore the test chamber for 10 min (sociability test). Another unfamiliar mouse (stranger 2, wild-type male) was placed in the cage that was empty in the sociability test. The subject was then allowed to explore the chamber again for 10 min (social novelty test). The time spent sniffing each wire cage was measured.

Resident–intruder test

The social behavior of the mutant mice was analyzed by a resident–intruder test ($n = 9$ for wild-type mice, $n = 10$ for knockout mice). An intruder male mouse (wild type, 88–95 % of the body weight of the test mouse) was placed into the home cage of the test mouse for 10 min under dim light. The time of contact between the resident and intruder mice was recorded. The behavior of the subject in response to the intruder was categorized as active, passive, or aggressive behavior.

Olfactory test

To test olfactory sensitivity, the mouse was deprived of food for 16 h and then placed in a test cage in which a piece of food was hidden under wood chips. The subject (n = 18 for each genotype) was allowed to explore in the cage for 5 min. The latency to finding the food was measured.

Exploratory test and open field test

Exploratory activity test was performed according to previously published methods (Huang *et al*., 2006). The subjects (n = 9 for wild-type mice, n = 10 for knockout mice) were individually habituated to an open field box (40 cm×40 cm×40 cm) with light of less than 10 lux in a 10-min session for 3 days. During the habituation session on the first day, walking path length and time spent on the center square were measured to evaluate anxiety level (open field test). During the test session, a small 5-cm square cube was placed in the center of the box, and the subject was allowed to explore the object for 10 min. The time spent sniffing the object was measured.

Elevated plus maze test

With some modification of the method by Moy *et al*. (2007), the elevated plus maze test was performed using a plus maze with two closed arms that had walls 40-cm in height, and two open arms, under dim light condition. The arms are 30 cm long and 5 cm wide, and the maze was elevated 50 cm from the floor. The subject (n = 9 for wild-type mice, n = 10 for knockout mice) was placed at the center of the maze and allowed to explore for 10 min. The time on and number of entry onto the open and closed arms were measured.

Light/dark box test

The light/dark box test was performed with some modification of a previously published method (Chen *et al*., 2006). The test apparatus is a rectangular Plexiglas box divided by a partition into two compartments. One compartment (17×28×28 cm) is left dark (<10 lux) and another one is lit with an overhead light (> 120 lux). The compartments were connected by an opening (7.5×7.5 cm) located at the floor level in the center of the partition. The subject (n=18 for each genotype) was placed into the light compartment and allowed to explore freely for 5 min. The time spent with all four paws in each compartment was measured.

Golgi staining

Golgi-Cox staining was performed according to Gibb and Kolb (1998). The analyses were performed three times independently using brains from N3 (16 week-old, n = 3 for wild-type mice, n = 3 for knockout mice), N6 (24 week-old, n = 3 for wild-type mice, n = 3 for knockout mice), and N10 (12 week-old, n = 4 for wild-type mice, n = 4 for knockout mice) naïve male mouse. Twenty to sixty of pyramidal neurons at the hippocampal CA1 region and cingulate cortex of each mouse were measured.

The detection of CREB phosphorylation

Mice were perfused transcardially with 4% paraformaldehyde 90 min after stimulation or stress-producing activity. Immunohistochemistry was performed as described previously (Mitsui *et al*., 2007b). In brief, the brain was sliced 22 μm thick, and was incubated with anti-CREB (86B19) mouse monoclonal antibody (Cell Signaling Technology Inc., Danvers, MA) and rabbit anti-phosphorylated CREB (S-133) antibody (Applied Biological Materials Inc., Richmond, Canada). Immunoreactivity was detected using anti-mouse IgG labeled with Alexa594 and anti-rabbit IgG labeled with Alexa488 (Invitrogen, Carlsbad, CA). The image was taken using a laser microscope (Eclipse 80i, Nikon, Tokyo, Japan) and a cooled CCD

camera (VB-7000, Keyence Corp., Osaka, Japan). The intensity of the immunoreactivity was measured by a MacSCOPE image analysis system (Mitani Corp., Tokyo, Japan).

Hippocampal activation by social interaction

Naïve mice (male, $n = 5$ for each genotype) were used to measure hippocampal activation by social interaction. They were group-housed after weaning and each was kept individually with a hetero mouse (a cage-mate) at least 2 weeks before the test. The subject and a cage-mate in their home cage were placed on a stage for 4 h to habituate to the stage environment. The cage-mate was then moved into a new cage. After 10 min, the cage-mate was placed in the home cage with the subject for 10 min, and then taken away again. Ninety minutes later, the brain of the subject mouse was prepared to investigate CREB phosphorylation.

Hippocampal activation by injecting pentylenetetrazol

Pentylenetetrazol (PTZ, Sigma Chemical Co., St. Louis, MO) was dissolved in PBS and was administered intraperitoneally at a dose of 50 mg/kg. Each subject was immediately placed in a clean bucket and was videotaped for 15 min ($n = 16$ for each genotype). The seizure events were defined as partial clonus (PC, partial or focal seizure lasting 1 or 2 sec), generalized clonus (GC, sudden loss of upright posture, whole body clonus lasting 30 – 60 sec), or tonic–clonic seizure (TC, maximum seizure characterized by hindlimb extension). Seizure score was calculated according to Ferrano *et al*. (1999) using the following equation:

$$\text{Seizure score} = 0.2 \times (1/\text{PC latency}) + 0.3 \times (1/\text{GC latency}) + 0.5 \times (1/\text{TC latency})$$

CREB phosphorylation in the hippocampus of mice after seizure induction were also performed as described above ($n = 4$ for wild-type mice, $n = 3$ for knockout mice).

Hippocampal activation by restraint stress

Restraint stress was conducted by placing the subject in a 50 mL Falcon tube for 2 hr as described by Kwon *et al*. (2007) ($n = 6$ for wild-type mice, $n = 7$ for knockout mice). The tube was placed in a home cage in order to avoid the change of stimuli from foreign odors and changes in ambient brightness that may affect the response to the restraint stress. Adequate ventilation was provided by holes at the bottom of the tube. Blood sample was prepared from the left cardiac ventricle immediately after restraint stress. Plasma cortisol was measured using radioimmunoassay by Mitsubishi Chemical Medience Corp. (Tokyo, Japan). The brains were prepared as described above for investigating CREB phosphorylation.

Hippocampal response to social isolation stress

To investigate the effect of social isolation during the social interaction test on CREB phosphorylation, no cage mate was presented to the subject once after it was moved into a new cage ($n = 4$ for each genotype). Other handling and the preparation of CREB phosphorylation was measured as described above.

Hippocampal responses to water stress

To eliminate a possibility that water stress during the water maze affected CREB phosphorylation in the hippocampus, CREB phosphorylation was investigated after water stress. The subjects ($n = 6$ for wild-type mice, $n = 4$ for knockout mice) were forced to swim for 60 sec, 4 times within one day, in the same circular pool used for the Morris water maze test with water made opaque, but without the platform. No landmarks were presented around

the pool. The swimming session was scheduled in the same way as the Morris water maze test including a probe trial. The brains were prepared 90 min after the final swimming session.

Data analysis

The data were presented as means ± SEM and analysed using a JMP software (SAS Institute Inc., Cary, NC). Statistical comparisons were made by Student's *t* test, ANOVA, and Tukey–HSD *post hoc* tests for pairwise comparisons. *P* values of less than 0.05 were considered significant.

Results

Generation of motopsin knockout mice and motor behavior characterization

The loss of motopsin in homozygous mutant mice was confirmed by northern hybridization and immunohistochemical staining in addition to Southern hybridization (see Fig. S1 in Supporting information). Motopsin mRNA or protein was detected in the wild-type and heterozygous mouse brain, but not in the motopsin knockout mouse brain. Homogeneous mutants appeared to grow normally, were fertile in both sexes, and lived for more than 18 months. Nissl staining did not reveal any gross anatomical abnormality in the brain. Beamwalking and footprint tests did not detect motor deficit in the mutant mice (Fig. S2 in Supporting information).

Normal memory formation of motopsin knockout mice

Because some MR patients have learning deficit, the spatial learning ability of motopsin knockout mice was investigated using a water maze test, passive avoidance test, and Y-maze test. In a hidden version of the Morris water maze test, the spatial learning ability of motopsin knockout mice was equivalent to wild-type mice during training sessions (Fig. 1A, two-factor ANOVA with repeated measure, main effect of genotype: $F_{1, 24} = 2.53$, $P = 0.125$; main effect of training day: $F_{4, 96} = 21.02$, $P < 0.0001$; interaction between effects of genotype and training day: $F_{4, 96} = 0.291$, $P = 0.883$). In a probe test, which was carried out 24 h after the final training trial, there was no difference in the swimming distance between both genotypes. Statistical analysis did not indicate differences in the amount of swimming time within the target quadrant between knockout and wild-type mice (Fig. 1B, two-factor ANOVA, main effect of genotype: $F_{1, 25} = 0.972$, $P = 0.334$; main effect of swimming zone, $F_{3, 75} = 31.86$, P < 0.0001), however, it revealed a significant interaction between effects of genotype and swimming zone ($F_{3, 75} = 3.105$, $P = 0.032$). *Post hoc* analysis indicated that amount of swimming time in the target quadrant of all mice was higher than those in other quadrants, but significant difference between genotypes was not detected. The analysis using swimming distance in the probe test showed similar findings (Fig. S3 in Supporting information).

In the passive avoidance test, there is no significant difference in the latency to enter the dark chamber between knockout and wild-type mice 1 day after training (Fig. 1C, $t_{26} = -1.92$, $P = 0.066$). Even 9 days after training, no difference was observed between two genotypes (Fig. S4B in Supporting information).

The Y-maze test assesses working memory based on the tendency of the mouse to explore a novel environment. Alternation was defined as entries into three different arms on consecutive choices. No obvious difference was found between the mutant and wild-type mice in percentage of alternation (Fig. 1D).

To assess the social memory of motopsin knockout mice, we performed a social recognition test. Repetitive presentations of the same mouse declined the sniffing time toward a stranger mouse in both the motopsin knockout and the wild-type mice (Fig. 2A, two-factor ANOVA, main effect of session, $F_{4, 100} = 12.33$, $P < 0.0001$; trial 2 versus trial 3 and 4, $P < 0.05$, *post hoc* analysis). The sniffing time then recovered when an unfamiliar mouse was presented (trial 4 versus trial 5, $P < 0.05$, *post hoc* analysis), indicating that motopsin knockout mice were capable of recognizing the new mouse was unfamiliar. Compared with wild-type mice, motopsin knockout mice appeared to show a longer sniffing time in response to both stranger mice, but the difference was not statistically significant (two-factor ANOVA, main effect of genotype: $F_{1, 25} = 0.38$, $P = 0.089$; interaction between effects of genotype and trial: $F_{4, 100} = 0.063$, $P = 0.819$).

Enhanced social interaction of motopsin knockout mice

Since social interaction in rodents depends greatly on olfactory information, we assessed the rodents' social interest by analyzing their olfactory behavior toward another mouse in a social behavior test. In a sociability test, both the wild-type and the motopsin knockout mice spent more time sniffing the cage containing an unfamiliar mouse (stranger 1) than the empty cage (Fig. 2B, two-factor ANOVA, main effect of genotype: $F_{1, 16} = 0.00$, $P = 1.00$; main effect of object: $F_{1, 16} = 566.29$, $P < 0.0001$; interaction between effects of genotype and object: $F_{1, 16} = 0.063$, $P = 0.805$), suggesting normal sociability of the mutant mouse. In a social novelty test, both mutant and wild-type mice spent more time sniffing the new unfamiliar mouse (stranger 2) than the other now-familiar mouse (stranger 1) they were acclimated to (Fig. 2C, two-factor ANOVA, main effect of object, $F_{1, 16} = 15.45$, $P = 0.001$). However, the mutant mice spent more time sniffing the familiar mouse (stranger 1) than the wild-type mice did (main effect of genotype, $F_{1, 16} = 7.21$, $P = 0.016$; interaction between effects of genotype and object: $F_{1, 16} = 0.323$, $P = 0.578$), suggesting that the mutant mice had enhanced social interest in even a familiar mouse.

To investigate the quality of social behavior in motopsin knockout mice, a resident-intruder test was performed. Motopsin knockout mice had significantly prolonged total interaction ($t_{17} = -2.48$, $P = 0.024$), although there was no difference in the time exhibiting any specific behavior that were active, passive, or aggressive (Fig. 2D).

Normal anxiety and exploratory activity of motopsin knockout mice

Various factors such as olfactory insensitivity, higher exploratory and locomotor activities, and decreased anxiety level may contribute to abnormal social behavior. To eliminate the possibility that the significant differences in social interactions of the knockout mice in comparison to their wild-type littermates mentioned above were due to any of these factors, we evaluated these behaviors in both groups. In an olfactory test, the mutant mice did not show a significant difference from wild-type mice in the latency to find the food buried under wood chips (Fig. 3A, $t_{34} = 1.54$, $P = 0.132$). Interestingly, the mutant mice showed a significant decrease in the latency to eat the food once they had found it ($t_{34} = 2.48$, $P = 0.018$).

Exploratory activity was investigated by measuring the latency and the time spent investigating a small object (e.g. a small cube). The mutant mice spent as much time sniffing the object as wild-type mice did (Fig. 3B). The latency to the first sniff was not different between genotypes.

Elevated plus-maze, light/dark box, and open field tests indicated similar activity level and anxiety level between motopsin knockout mice and wild-type mice (Fig. 4). An open field test failed to detect any difference of distance moved between the genotypes, indicating that

motopsin knockout mice have normal locomotor activity. In summary, olfactory acuity, exploratory activity level, locomotor activity, and anxiety level of the mutant mice were comparable to those of wild-type mice, suggesting motopsin preferentially affects social behavior.

Impaired hippocampal function by motopsin-deficit

Golgi-Cox staining revealed that motopsin knockout mice had decreased spine density on apical dendrites (t_7= 2.80, P = 0.0265), but not on basal dendrites, of the hippocampal CA1 region (Fig. 5). This defect of synaptic structure has been reported in some MR and is thought to be involved in cognitive deficit (Chechlacz & Gleeson, 2003). Spine density alteration appears to be present in the hippocampus and absent in the cingulate cortex.

To further explore the functioning of the hippocampus in motopsin knockout mice, we investigated the activation of hippocampal neurons after behavioral stimulation by measuring the phosphorylation of CREB at Ser133, which is known to be a key event for neuronal plasticity. First, after each mouse was trained on the Morris water maze, the ratio of phosphorylated CREB and total CREB proteins were immunohistochemically assessed. The ratio of phosphorylated CREB to total CREB protein was significantly decreased in the mutant mice compared to the wild-type mice (t_6 = 2.86, P = 0.029, Fig. 6 A, B). We then investigated the effect of social stimulation on CREB phosphorylation. To exclude influences other than social stimulation, the subject was presented with a cage-mate that had been kept together with the subject since weaning. Phosphorylated CREB was detected in the CA1 hippocampal neurons of wild-type mice, whereas it was barely detected in the motopsin knockout mice (t_8 = 4.69, P = 0.002, Fig. 6C). As a control measurement to ensure that knockout mice did in fact have the capability to complete CREB phosphorylation, intraperitoneal injection of convulsant reagent, pentylenetetrazol, and subsequent measure of CREB phosphorylation was done. Motopsin knockout mice responded to the reagent with phosphorylation of CREB at a level compatible to that of wild-type mice, which was consistent with the fact that mutant mice showed similar latency and severity of the seizure to that by wild-type mice (Fig. 7).

In order to exclude the possibility that the difference of CREB phosphorylation between genotypes is derived from different sensitivity against stresses, we investigated CREB phosphorylation after various stress-dependent behaviors including social isolation stress, water stress, restraint stress, passive avoidance test. Social isolation stress slightly induced CREB phosphorylation in the hippocampus of both wild-type and mutant mice, but the degree of induction did not differ between the two genotypes (Fig. S5A in Supporting information). There is no difference between genotypes in CREB phosphorylation induced by water stress (Fig. S5B in Supporting information). Furthermore, restraint stress increased plasma cortisol level in both genotypes and induced CREB phosphorylation in the hippocampus to the same degree (Fig. S6 in Supporting information). After the passive avoidance test, the ratio of pCREB/CREB was similar in each genotype (Fig. S4C in Supporting information). These control experiments suggest that CREB phosphorylation is attributed to social interaction with a stimulator mouse and the retrieval of spatial memory.

In summary, motopsin knockout mice retained their ability to undergo CREB phosphorylation, but without motopsin, hippocampal neurons did not complete activity-dependent phosphorylation of CREB in some hippocampal-dependent behaviors.

Discussion

A mutation of motopsin gene, which causes an immature stop codon, has been implicated in MR (Molinari *et al*., 2002). The knockdown of the expression of *Drosophila* tequila, the

ortholog of motopsin, has been found to impair long-term memory formation, but not short-term memory (Didelot *et al*., 2006). However, in this study, memory deficit was not detected in mice with an absence of motopsin protein in tests such as passive avoidance test, Y-maze test, or social memory test. The Morris water maze for spatial memory revealed virtually no differences in the behavior of mice lacking motopsin. This is not entirely surprising, since the Morris water maze test appears to not be particularly sensitive in detecting impaired spatial memory in mice lacking a mental retardation gene. It has failed to reveal differences in other lines such as FMR1, Gdi1, and Pak3 knockouts while other memory tests could detect cognitive deficits in them (Paradee *et al*., 1999; D'Adamo *et al*., 2002; Meng *et al*., 2005).

MR defined as an overall intelligent quotient below 70 is generally accompanied by a functional deficit in adaptive behavior such as social skills and communication. For example, MR patients who have Down's and Williams syndrome frequently show higher social interest or familiarity (Mervis & Klein-Tasman, 2000; Visootsak & Sherman, 2007). It is unknown, however, whether individuals with motopsin deficit demonstrate the same tendency. Our motopsin knockout mice suggests that motopsin deficiency may have the same effect on social interest. In the social novelty test, knockout mice had a higher interest in the familiar mouse than wild-type mice did, and they showed prolonged interactions with an intruder mouse in the resident-intruder test. This difference is most likely highly specific without interference by attributes such as changes to olfactory acuity, exploratory activity, anxiety level and locomotor activity, which were all unaffected in our knockout mice. This abnormal social behavior of the motopsin knockout mice suggests another role of this protease in the mammalian cognitive function.

Our motopsin knockout mice may be useful for understanding the neuronal mechanism of social behavior and possibly for the analysis of abnormal social behavior of some MR patients. The abnormal social behavior in mice lacking other MR-related genes accompanies enhanced anxiety level (Spencer *et al*., 2005; Kwon *et al*., 2006) or reduced aggressive behavior (D'Adamo *et al*., 2002). Unlike other MR model mice, motopsin knockout mice showed normal anxiety levels in three independent tests including the elevated plus-maze test, light/dark box test, and open field test. Furthermore, our motopsin knockout mice showed prolonged social interaction while most rodent MR models show impaired social interactions. This prolonged social interaction suggests that motopsin may preferentially affect social behavior rather than emotional behavior. The result of the social recognition test and increased sniffing time of unfamiliar mice longer than the familiar mice seen with our knockout mice indicate the ability of the mutant mouse to recognize and discriminate stranger mice. Histologically, motopsin knockout mice showed decreased spine density in the hippocampal CA1 region, but not in the cingulate cortex. This regional differential effect may be explained by the fact that motopsin is more highly expressed in the hippocampal CA1 region than the cingulate cortex in adult mouse brain (Iijima *et al*., 1999; Mitsui *et al*., 2007b). Abnormal spine density is common in some MR patients and models of Fragile X syndrome (Irwin *et al*., 2000), Down syndrome (Belichenko *et al*., 2004), and Rett syndrome (Belichenko *et al*., 1994; Armstrong, 2005). Alterations of target genes responsible for these diseases impair neuronal plasticity (Kleschevnikov *et al*., 2004; Asaka *et al*., 2006; Moretti *et al*., 2006). Mice lacking an MR gene, such as Gdi1 or p21 activating kinase 3 gene, exhibit a defect of hippocampal long-term potentiation (D'Adamo *et al*., 2002; Meng *et al*., 2005). We showed that in mice lacking motopsin, one component of neuronal plasticity, CREB phosphorylation in hippocampal CA1 neurons that was typically induced by memory formation and social interaction, was markedly reduced. Even while behavioral differences were not discerned in the Morris water maze test, a dramatic inhibition of CREB phosphorylation was seen in knockout mice after this test. This reduction in phosphorylation is most likely specific to memory formation rather than to a difference in response to simply

water stress, since no difference in CREB phosphorylation between wild-type and mutant mice were seen after the water stress test.

Abnormal social behavior is most likely associated with the deficit of hippocampal function in motopsin knockout mice because the hippocampus is known to be crucial for not only memory formation, but also social behavior. A lesion of the hippocampus caused by ibotenic acid disrupts social memory (Kogan *et al*., 2000). Alteration of many genes expressed in the hippocampus have been found to disrupt social behavior (D'Adamo *et al*., 2002; Kwon *et al*., 2006; Moretti *et al*., 2006; Nishijima *et al*., 2006; Morellini *et al*., 2007). The hippocampal region is also necessary for the recognition of familiar individuals (Lai *et al*., 2005). In this report, we showed that motopsin knockout mice have a decreased response in hippocampal neurons after social interactions with their cage-mate.

Activity-dependent secretion from hippocampal neurons and capture of motopsin around synapses have been reported (Frischknecht *et al*., 2008). We further know that motopsin digests a proteoglycan, agrin, which regulates synapse formation in the central nervous system (Reif *et al*., 2007; McCroskery *et al*., 2006; Ksiazek *et al*., 2007; Stephan *et al*., 2008). Recently, it was reported that motopsin-dependent agrin digestion is necessary for long-term potentiation-induced formation of filopodia, which is believed to be an immature spine structure in post-synaptic neurons (Matsumoto-Miyai *et al*., 2009). Surprisingly in this study the authors showed that despite this, motopsin-deficiency shows no effect on long-term potentiation at the hippocampus. These findings are consistent with our data showing that the deficiency of motopsin causes dramatic cellular changes but less observable functional changes, such as long-term potentiation and behavioral phenotypes. We summarized the behavioral phenotype and corresponding CREB phosphorylation in the hippocampus in table I. While the CREB phosphorylation results are more robust, we can certainly associate the decreased CREB phosphorylation in motopsin knockout mice directly with the detectable behavioral abnormalities. Thus, a lack of motopsin in the hippocampal region may underlie the observed alteration in hippocampal-dependent behaviors perhaps via agrin-dependent synapse formation.

More than 290 genes are involved in clinical phenotypes, syndromes, or neurological disorders characterized by MR (Inlow & Restifo, 2004; Chelly *et al*., 2006). Many of the characterized MR-related genes encode intracellular proteins that are categorized as transcriptional and chromatin-remodeling factors, microtubule- and actin-associated proteins, or effectors of RhoGTPase pathways. In contrast, motopsin is the first known extracellular serine protease that is linked to MR. Recently, other extracellular serine proteases have been implicated in the modulation of neuronal plasticity (Yoshida & Shiosaka, 1999; Shiosaka & Yoshida, 2000; Tomimatsu *et al*., 2002; Mitsui *et al*., 2007a). For example, tissue plasminogen activator (tPA) contributes to long-term potentiation by cleavage of the precursor form of brain-derived neurotrophic factor and interacts with the *N*-methyl-D-aspartate receptor (Nicole *et al*., 2001; Pang *et al*., 2004). Neuropsin is also known to affect the formation of excitatory synapses in the hippocampal CA1 region and modulate neuronal plasticity by cleaving the presynaptic adhesion molecule L1 (Hirata *et al*., 2001; Matsumoto-Miyai *et al*., 2003). The lack of either tPA or neuropsin gene impairs memory formation. However, it is still unknown whether these proteases are involved in social behavior. Our findings indicate for the first time that extracellular proteolysis in the central nervous system contributes not only to memory formation, but also to higher cognitive function such as social interest. Future research should focus on the mechanism of activation and localization of secreted motopsin which would further our understanding of the molecular mechanism of motopsin's function in neurological disorders such as MR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CREB	cAMP responsive element binding protein
IR	immunoreactivity
MR	mental retardation
tPA	tissue plasminogen activator

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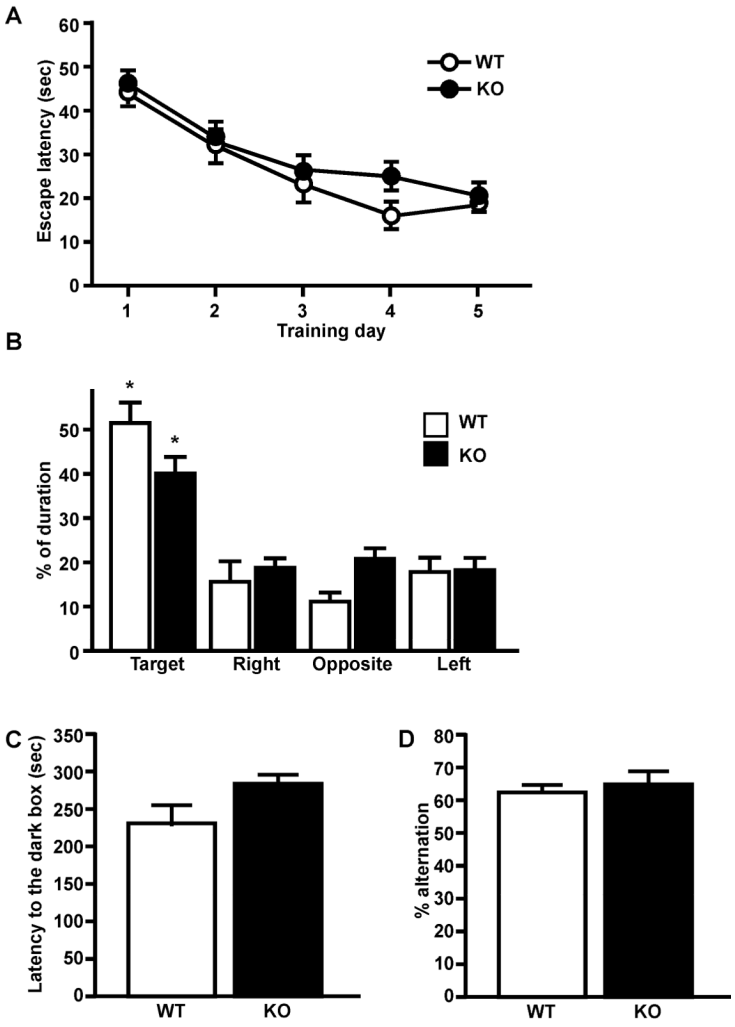

FIG.1.

Motopsin knockout mice showed normal spatial memory. (A) Training session of Morris water maze test. Knockout (KO) mice showed spatial learning ability comparable to wild-type (WT) mice. Open circles, WT; Closed circles, KO. (B) Probe test. Both wild-type and motopsin KO mice spent a longer time swimming within the target quadrant compared to other quadrants 24 h after their final training session (* $P < 0.05$, *post hoc* analysis after a two-factor ANOVA). (C) Passive avoidance test. Motopsin KO mice showed similar latency to enter the dark box as WT mice 24 hr after the training. (D) Y-maze test. KO mice showed a proportion of alternation similar to that by WT mice in the Y-maze test. Values represent mean ± SEM.

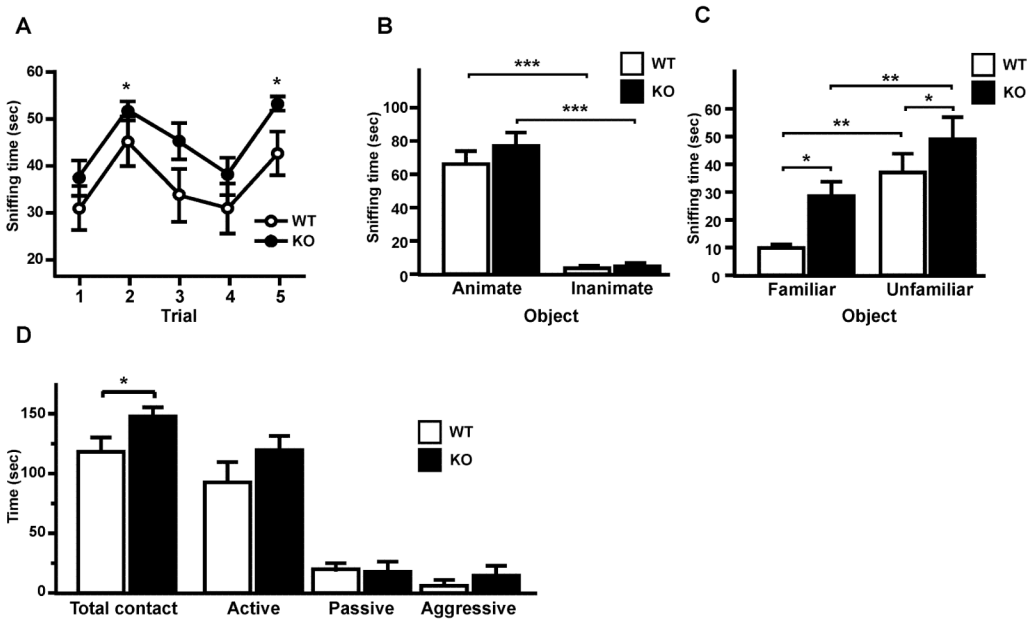

FIG. 2.

Abnormal social behavior in motopsin KO mice. Motopsin KO mice showed the ability to discriminate between familiar and unfamiliar mice; however, they investigated even a familiar mouse for longer than WT mice did. Values represent mean ± SEM. (A) Social recognition test. KO mice recognized the stranger mice, which is shown by the decline in sniffing time over the first four trials when they were presented with the same stranger mouse. When KO mice were exposed to an unfamiliar mouse at trial 5, the investigation time recovered to that at trial 2 which was significantly different from the investigation time of trial 4 (*$P < 0.05$, *post hoc* analysis after a two-factor ANOVA). (B) Sociability test. Both WT and KO mice sniffed the unfamiliar mouse significantly longer than the empty cage (***$P < 0.0001$), indicating that motopsin KO mice had normal sociability. (C) Social novelty test. Both WT and KO mice preferred the unfamiliar mouse to the familiar mouse (**$P = 0.001$). However, KO mice spent more time sniffing the familiar mouse than WT mice did (*$P < 0.05$). (D) Resident–intruder test. KO mice showed longer contact time than WT mice (*$P < 0.05$). However, the duration of active, passive, or aggressive behavior was not different between the genotypes.

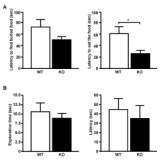

FIG. 3.

Normal olfactory behavior and exploratory activity in motopsin KO mice. (A) Olfactory test. Motopsin KO mice spent as much time finding buried food as WT mice (left). Motopsin KO mice ate more quickly than WT mice (right) (*$P < 0.05$). (B) Exploration test. Time spent investigating a novel object (left) or latency to the first investigation (right) was not different between the genotypes. Values represent mean ± SEM.

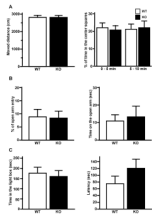

FIG. 4.

Normal anxiety level of motopsin knockout mice. (A) Open field test. The mutant mice moved as much as WT mice (left). There is no difference between the genotypes in the proportion of distance passed in the center squares during 5 min sessions (right). (B) Elevated plus maze. There was no difference between genotypes in frequency of open arm entry (left) and time spent on open arms (right). (C) Light/dark box test. Time spent in the bright chamber (left) and latency to the first entry into the dark chamber (right) was not significantly different between WT and KO mice. Values represent mean ± SEM.

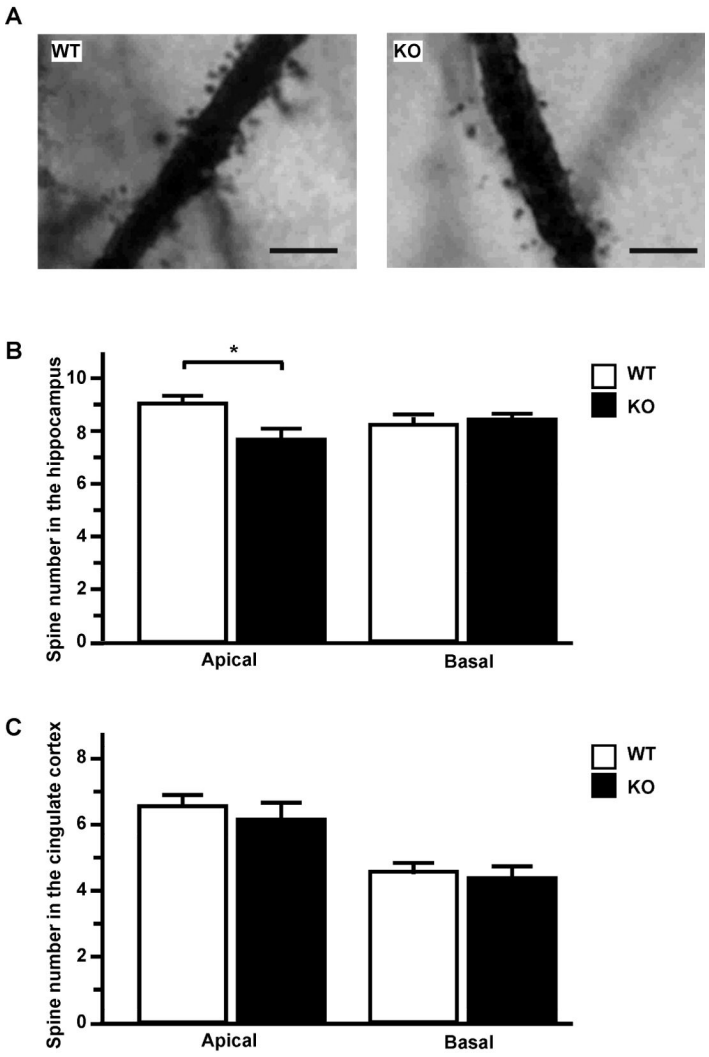

FIG. 5.

Decreased spine density of apical dendrites in hippocampal neurons but not in cortical neurons. (A) The morphology of dendritic spines was not different between WT and KO mice, but the spine number appeared to be decreased in motopsin KO mice. Bar, 10 μm. (B) Spine density (per 20 μm) on apical dendrites of pyramidal neurons in the hippocampus was decreased in motopsin KO mice (**P* < 0.05, n = 4 mice for each genotype), although the spine density on basal dendrites did not differ between the genotypes. The analyses were independently performed three times using N4, N6, and N10 in mouse brains. Declines were consistent across all generation groups tested. (C) There was no difference in the spine density of pyramidal neurons in the cingulate cortex between the genotypes. Values represent mean ± SEM.

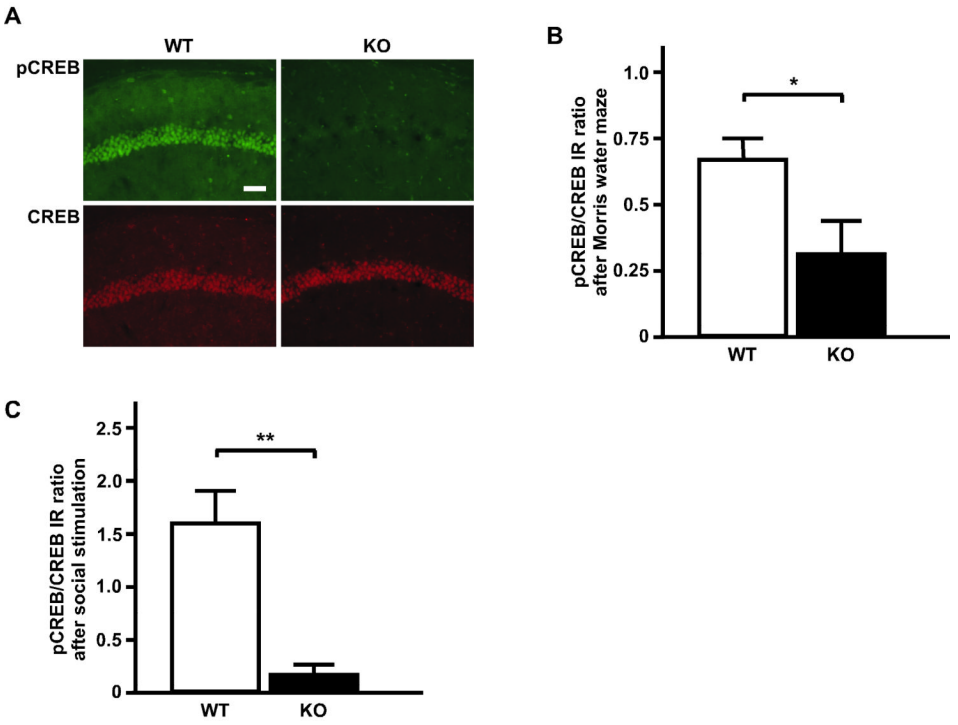

FIG. 6.

Decreased response in hippocampal neurons of motopsin knockout mouse to memory formation or social stimulation. (A) Immunohistochemistry using anti-CREB phosphorylated at Ser133 (pCREB) and anti-CREB. Hippocampal neurons were strongly stained by anti-pCREB antibody in WT mice, but barely in motopsin KO mice 90 min after the water maze training. Bar, 50 μm. (B) The ratio of fluorescence intensity of pCREB immunoreactivity (IR) to CREB IR after the water maze training. The ratio of pCREB/CREB IR in the hippocampus was significantly decreased in motopsin KO mice compared with WT mice (*$P < 0.05$). (C) After social stimulation, the pCREB/CREB IR ratio also significantly decreased in the motopsin KO mice (**$P < 0.01$).

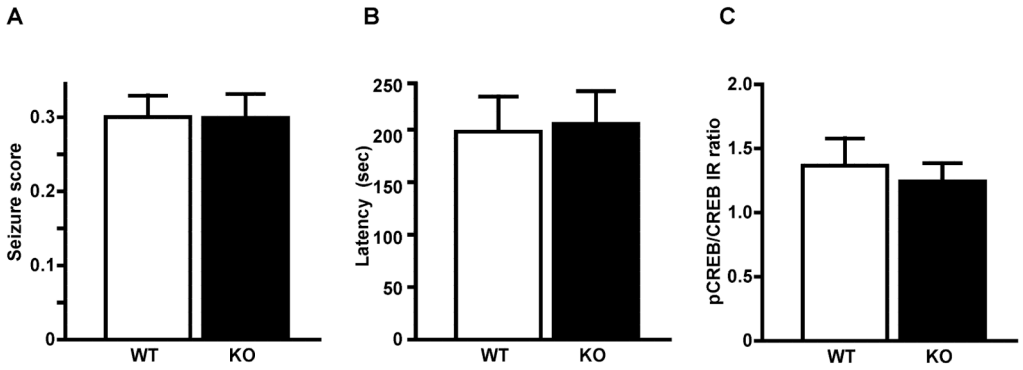

FIG.7.

Behavioral phenotype and CREB phosphorylation to PTZ administration. (A) Seizure score. The score was calculated in consideration of the severity and latency of seizure using an equation stated in Materials and Methods. The score of motopsin knockout mice was similar to that of wild-type mice. (B) The latency to tonic-clonic seizure. There is no difference between genotypes in the latency to tonic-clonic seizure after administration of PTZ. (C) High level of CREB phosphorylation was induced by PTZ administration at hippocampal neurons of both wild-type and mutant mice.

Table 1

CREB phosphorylation and behavioral phenotype

Type of behavior	Phenotype of KO mice	CREB phosphorylation	
		WT	KO
Social interaction	Enhanced	↑↑↑	±
Water maze	Mildly affected	↑↑	↑
Passive avoidance	n.s.	↑	↑
PTZ seizure	n.s.	↑↑↑	↑↑↑
Restraint stress	n.s.	↑	↑

n.s., The difference in behavioral phenotype was not significantly detected.