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RacGAP α2-Chimaerin Function in Development Adjusts Cognitive Ability in Adulthood

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SUMMARY

A major concern in neuroscience is how cognitive ability in adulthood is affected and regulated by developmental mechanisms. The molecular bases of cognitive development are not well understood. We provide evidence for the involvement of the α2 isoform of Rac-specific guanosine triphosphatase (GTPase)-activating protein (RacGAP) α-chimaerin (chimerin) in this process. We generated and analyzed mice with global and conditional knockouts of α-chimaerin and its isoforms (α1-chimaerin and α2-chimaerin) and found that α-chimaerin plays a wide variety of roles in brain function and that the roles of α1-chimaerin and α2-chimaerin are distinct. Deletion of α2-chimaerin, but not α1-chimaerin, beginning during early development results in an increase in contextual fear learning in adult mice, whereas learning is not altered when α2-chimaerin is deleted only in adulthood. Our findings suggest that α2-chimaerin acts during development to establish normal cognitive ability in adulthood.

INTRODUCTION

Cognitive ability in adulthood is affected by developmental mechanisms (Tau and Peterson, 2010). The molecular bases for this process are not well understood. Recent evidence obtained primarily from studies of developmental psychiatric disorders, such as autism spectrum disorders (ASDs) and schizophrenia, suggests that Rho family small guanosine triphosphatases (Rho GTPases) and their regulators could be involved in this process (Ba et al., 2013; Cahill et al., 2009; Carlson et al., 2011; Ramakers et al., 2012; van Bokhoven, 2011). Rho GTPases, such as RhoA, Rac, and Cdc42, are key regulators of actin dynamics, and they are activated by Rho guanine nucleotide exchange factors (GEFs) and inactivated by Rho GTPase-activating proteins (GAPs) (Luo, 2000). However, because most studies rely on phenotypes of global knockout (KO) mice (Carlson et al., 2011; Ma et al., 2008; Oh et al., 2010), the significance of developmental Rho GTPase signaling for adult cognition is not fully understood.

α-chimaerin (α-chimerin) is a Rac-specific GAP (RacGAP) expressed specifically in the nervous system (Diekmann et al., 1991; Hall et al., 1990, 1993). α-chimaerin is composed of α1 and α2 isoforms (α1-chimaerin and α2-chimaerin, respectively), which are transcribed from distinct promoters (Dong et al., 1995; Hall et al., 1990, 1993). The α1 isoform is dominantly expressed in adulthood, whereas the α2 isoform is dominantly expressed during development (Hall et al., 2001; Lim et al., 1992). Studies in the *miffy* mouse, a spontaneous *α-chimaerin* (*αChn*) null mutant mouse, and an *αChn* KO mouse have revealed an in vivo function for α-chimaerin (Iwasato et al., 2007). α2-chimaerin regulates midline axon guidance of the corticospinal tract (CST) and the central pattern generator (CPG), and a loss of function of this protein results in hopping gaits in the animals. Subsequently, three other groups reported similar results using different sets of KO mice (Beg et al., 2007; Wegmeyer et al., 2007) or in vitro models (Shi et al., 2007). A recent study using a knockdown approach suggests a role for α2-chimaerin in cortical neuron migration, albeit in a RacGAP-independent manner (Ip et al., 2012). In humans, gain-of-function mutations of the α-chimaerin gene *CHN1* cause Duane's retraction syndrome, an eye movement disorder caused by the disruption of axon guidance in the ocular motor system (Miyake et al., 2008). These reports suggest important roles of α-chimaerin in axonal development and neuronal migration; however, the roles of α-chimaerin in higher brain function are unexplored.

By generating a series of mice with global and conditional KO of α-chimaerin and its isoforms and analyzing them, we found that deletion of α2-chimaerin from the developmental stages, but not in adulthood, results in an increase in contextual fear learning. These findings suggest that α2-chimaerin acts during development to establish normal cognitive ability in adulthood.

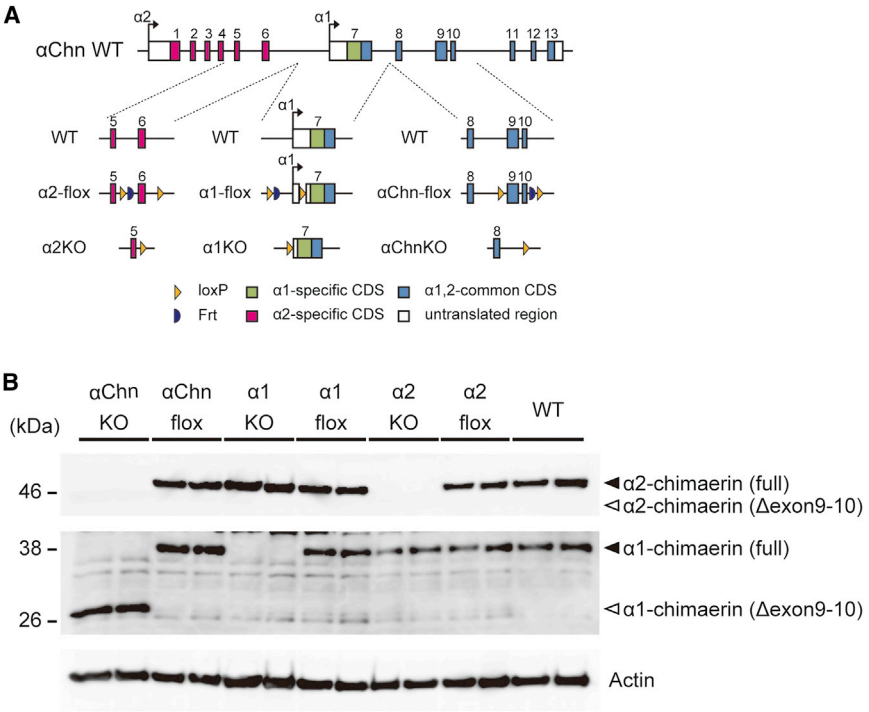

Figure 1. Generation of a Series of αChn-Flox and KO Mouse Lines

(A) Schematics for WT, isoform-specific and pan-isoform flox, and isoform-specific and pan-isoform KO alleles of αChn. In the α2 isoform-specific flox (α2-flox) allele, the exon 6 is flanked by two loxPs; this exon is deleted in the α2-specific KO (α2KO) allele. In the α1 isoform-specific flox (α1-flox) allele, the transcriptional initiation site of α1 isoform is flanked by two loxPs; this locus is deleted in the α1-specific KO (α1KO) allele. In the pan-isoform flox (αChn-flox) allele, exons 9 and 10, which code for the amino acids essential for RacGAP activity, are flanked by two loxPs. In the αChnKO allele, exons 9 and 10 are deleted. CDS, coding sequence.

(B) Western blot analyses of the postnatal day 14 (P14) hippocampus using α1-chimaerin-specific and α2-chimaerin-specifc antibodies. Normal levels of α1-chimaerin and α2-chimaerin (black arrowheads) were detected in the αChn-flox ($Chn1^{flox/flox}$), α2-flox ($Chn1^{\alpha 2flox/\alpha 2flox}$), and α1-flox ($Chn1^{\alpha 1flox/\alpha 1flox}$) mice. Only the truncated α1-chimaerin and α2-chimaerin (white arrowheads), which lack RacGAP activity (Iwasato et al., 2007), were detected in the αChnKO ($Chn1^{-/-}$) mice. α1-chimaerin, but not α2-chimaerin, was lacking in α1KO ($Chn1^{\Delta\alpha 1/\Delta\alpha 1}$) mice. α2-chimaerin, but not α1-chimaerin, was lacking in the α2KO ($Chn1^{\Delta\alpha 2/\Delta\alpha 2}$) mice.

RESULTS

Behavioral Abnormalities in Global αChn-Deficient Mice

To investigate the roles of α-chimaerin in the broader aspects of neural circuit development and function, we performed a comprehensive behavioral test on αChn-deficient mice ($Chn1^{mfy/mfy}$ or $Chn1^{-/-}$ [αChnKO] mice) and their littermate controls ($Chn1^{mfy/+}$ or $Chn1^{+/-}$, respectively) (Figure 1). In addition to the rabbit-like hopping gait (Iwasato et al., 2007), αChn-deficient mice exhibited abnormalities in various behavioral tests (Figures 2 and 3; Table S1). A striking phenotype was their extremely high levels of locomotor activity. These mice exhibited approximately 4- and 18-fold higher locomotor activity than the control mice in the open-field (Figure 2A) and home cage activity tests (Figure 2B), respectively. Another and more intriguing phenotype was observed in the contextual fear-learning test (Figures 3A and 3B). The fear-conditioning tests were applied to assess the subjects' learning and memory abilities (LeDoux, 2000). In these tests, αChnKO mice exhibited normal "freezing" levels in the conditioning phase (data not shown) and cued test (Figure 3Aa). In contrast, in the contextual test, which is a hippocampus-dependent task (Kim and Fanselow, 1992; Phillips and LeDoux, 1992), αChnKO mice exhibited increased "freezing" compared with the controls (Figure 3Aa), indicating enhanced contextual fear memory in these mice. In addition, we found that a *CHN1* polymorphism is associated with the autistic trait and arithmetic ability in healthy adult humans (Figure S1). These results imply that α-chimaerin has a role in cognition in both mice and humans. In the following studies, we primarily focused on contextual fear conditioning and other types of hippocampus-dependent learning in mice.

An Increase in Contextual Fear Learning in Dorsal Telencephalon-Specific αChnKO Mice

Because global αChn-deficient mice exhibited abnormalities in various behavioral paradigms (Table S1), it was unclear whether the increase in the contextual fear-learning phenotype was ascribed to the direct effects of the α-chimaerin disruption or was merely a consequence of other behavioral abnormalities such as locomotor hyperactivity and hopping gait. To distinguish between these two possibilities, we generated region-specific αChnKO mice using the Cre/loxP system. αChn-flox mice (Figure 1) were crossed with Emx1-Cre knockin mice, which exhibit Cre-mediated recombination in all excitatory neurons of the dorsal telencephalon (DT), including the hippocampus, amygdala, and cerebral cortex from the embryonic stages (Figure S2) (Iwasato et al., 2000, 2008), to generate DT-αChnKO ($Emx1^{Cre/+}$; $Chn1^{flox/-}$) mice. The DT-αChnKO mice and their littermate controls ($Chn1^{flox/-}$) were subjected to a series of behavioral tests. Although the DT-αChnKO mice behaved normally in most behavioral paradigms (Figures 2D and 2E; Table S1), they exhibited an increase in contextual fear-learning behavior similar to that observed in the global αChnKO mice (compare Figures 3Aa and 3Ab). These results demonstrate that an increase in contextual fear memory was caused by the direct effects of a loss of α-chimaerin in the excitatory neurons of the DT.

The α2 Isoform, but Not the α1 Isoform, Regulates Hippocampus-Dependent Learning

α1-chimaerin and α2-chimaerin exhibit different biochemical properties in vitro, and they have temporally distinct expression patterns in the mouse brain (Buttery et al., 2006; Hall et al., 2001; Lim et al., 1992). To determine whether these two isoforms have

distinct functions in vivo, we generated isoform-specific flox and KO mice (Figure 1) and subjected the isoform-specific KO mice to a series of behavioral tests. The α2 isoform-specific KO (α2KO) (*Chn1*$^{\Delta\alpha2/\Delta\alpha2}$) mice exhibited similar phenotypes as the αChn-deficient mice, in which both α1 and α2 isoforms were disrupted (Figures 2I, 2J, and 3Ad; Table S1). In contrast, the α1 isoform-specific KO (α1KO) (*Chn1*$^{\Delta\alpha1/\Delta\alpha1}$) mice exhibited distinct behavioral phenotypes (Figures 2F–2H and 3Ac; Table S1). For instance, in the elevated plus maze test, the α1KO mice exhibited increased anxiety-like behavior (Figure 2H), whereas α2KO mice exhibited no anxiety-like behavior (Figure 2J). These results suggest that the α1 and α2 isoforms play distinct roles in brain function. However, because α1 expression was lower in α2KO mice compared to in wild-type (WT) mice and α2 expression was higher in α1KO mice compared to in WT mice (Figure S3), crosstalks between isoforms might also be considered.

In the fear-conditioning test, the α1KO mice exhibited normal fear memory in both cued and contextual tests compared with their controls (*Chn1*$^{+/+}$) (Figure 3Ac). However, both α2KO and DT-specific α2KO (DT-α2KO [*Emx1*$^{Cre/+}$;*Chn1*$^{\alpha2flox/-}$]) mice exhibited an increase in contextual fear memory when compared to their controls (*Chn1*$^{\Delta\alpha2/+}$ and *Chn1*$^{\alpha2flox/-}$, respectively, Figures 3Ad and 3Ae). To confirm specificities of behavioral deficits of α2-chimaerin mutants further, we examined α2KO mice with the contextual discrimination test (Figure 3C). In this test, electrical footshock was applied to mice in a context (context A), and then freezing behavior was analyzed in a novel context (context B) and the conditioned context (context A). α2KO mice exhibited enhancement of freezing compared to control mice in the conditioned context, but not in the novel context or baseline. These results indicate that deletion of α2-chimaerin did not lead to an increase of basal levels of freezing or generalized fear but led to a context-dependent enhancement of fear learning.

We then assessed spatial memory, which is also hippocampus dependent, in the α2KO mice using an automated monitoring system for mouse behavior in a social context, named the ''IntelliCage'' system (Krackow et al., 2010; Voikar et al., 2010). In the avoidance-learning test, water is available for the mice to drink in all four corners of the cage; however, one corner is designated as the air-punishment corner. The α2KO mice exhibited a higher percentage of ''Visit Avoidance'' to the air-punishment corner in the probe trial compared with the controls (Figure 3Da), indicating that hippocampus-dependent avoidance memory is also increased in α2KO mice. In contrast, extinction learning was not different between the genotypes (Figure 3Db). In the place-preference test, mice were able to drink water in only one corner, whereas in the reversal-learning test, water was available only in the opposite corner (Figure 3Ea). The α2KO mice exhibited higher place preference (Figure 3Eb) and reversal learning (Figure 3Ec) than the control mice, although the differences were not significant. Thus, the α2KO mice exhibited enhanced hippocampus-dependent learning in both contextual fear learning and spatial avoidance learning in the IntelliCage system.

α2-Chimaerin in Adulthood Is Dispensable for Contextual Fear Learning

Because gene disruption occurs from the early developmental stages in both the α2KO and DT-α2KO mice, it is important to determine whether α2-chimaerin is involved in the negative regulation of hippocampus-dependent learning during development or adulthood. Although α2-chimaerin expression in the hippocampus was detected even in adulthood (e.g., at 2 months old), its levels were higher during development (Figures 4A and 4B) (Buttery et al., 2006; Hall et al., 2001), which suggests important roles of α2-chimaerin in development including juvenile stages (around 2–4 weeks old). To examine this, we generated temporally controlled α2KO mice. We crossed α2-flox mice with SLICK-H transgenic mice, which robustly express CreERT2 recombinase widely in the brain (Heimer-McGinn and Young, 2011), to obtain *SLICK-H*;*Chn1*$^{\alpha2flox/-}$ mice. Tamoxifen was administrated to 5- to 8-week-old *SLICK-H*;*Chn1*$^{\alpha2flox/-}$ mice for 10 days (see the Supplemental Experimental Procedures), and these mice were referred to as adult-specific α2KO (Ad-α2KO) mice. The fear-conditioning test was performed 1 month after the last administration of tamoxifen; at this stage, α2-chimaerin protein was barely detectable in the Ad-α2KO hippocampus (Figure 4C). However, in contrast to the α2KO and DT-α2KO mice, which exhibited an increase in contextual fear memory, Ad-α2KO mice exhibited normal fear memory in both the cued and contextual tests compared with their littermate controls (tamoxifen-treated *SLICK-H*;*Chn1*$^{\alpha2flox/+}$) (Figure 4D). In addition, we generated adult-specific αChnKO (Ad-αChnKO; tamoxifen-treated *SLICK-H*;*Chn1*$^{flox/-}$) mice and demonstrated that they also exhibited normal fear memory (data not shown). These results suggest that α2-chimaerin is involved in contextual fear learning in the developing brain (including the juvenile brain), but not in the adult brain. Taken together, our findings (Figures 3 and 4) revealed that α2-chimaerin acts in the DT during development and establishes normal hippocampus-dependent learning ability in adulthood.

DISCUSSION

In this study, we demonstrated that hippocampus-dependent learning, such as contextual fear learning, is enhanced in mice that lack the α2 isoform of α-chimaerin in the DT including hippocampus, amygdala, and cerebral cortex from the developmental stages (αChnKO, DT-αChnKO, α2KO, and DT-α2KO mice), but this enhancement was not observed in the α1KO or Ad-α2KO mice (Figures 3A–3D, 4, and S2). These results suggest that Rac-GAP α2-chimaerin is important for cognitive development.

Accumulating evidence obtained principally from studies of developmental psychiatric disorders, such as ASDs and schizophrenia, suggests that Rho GTPases (e.g., RhoA, Rac, and Cdc42) and their regulators (e.g., GEFs and GAPs) are involved in cognitive ability (Ba et al., 2013; Endris et al., 2002; Piton et al., 2011; Ramakers et al., 2012). However, because most of the previous studies rely on phenotypes of global KO mice (Carlson et al., 2011; Ma et al., 2008; Oh et al., 2010), the significance of developmental Rho GTPase signaling for adult cognition is not well understood. For example, global disruption of RacGEF kalirin-7 results in impaired acquisition of a passive avoidance task (Ma et al., 2008). However, because kalirin-7 functions in both synapse formation during development and spine plasticity in adulthood (Penzes and Jones, 2008), it is unclear if kalirin-7 during development or in adulthood is important for adult

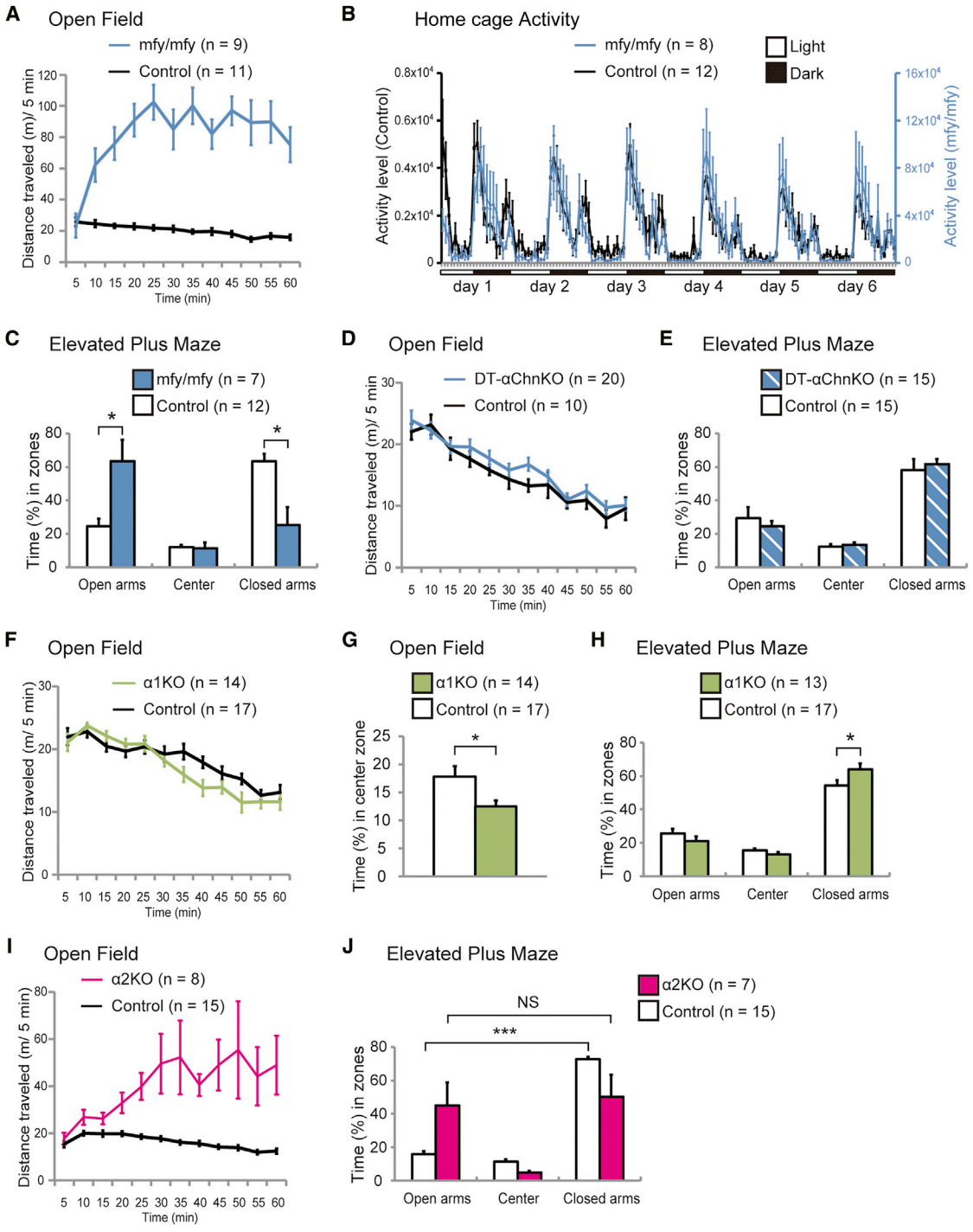

Figure 2. Representative Behavioral Phenotypes of a Series of α-Chimaerin Mutant Lines

(A) In an open-field test, total distance traveled of αChn-deficient mice was 4-fold greater than that of control mice (Control, 244.5 ± 17.6 m; *mfy/mfy*, 973.1 ± 93.3 m; $p < 0.001$ for genotype), although for the first 5 min, there was no significant difference between genotypes ($p > 0.05$).

(B) In the home cage activity test, αChn-deficient mice showed extremely increased locomotor activity assessed by the number of photobeam interruptions: (light cycle: Control, 7,009.7 ± 1,935.9 cases/day; *mfy/mfy*, 59,161.1 ± 24,896.4 cases/day, $p < 0.01$ for genotype; dark cycle: Control, 24,214.9 ± 5,459.1 cases/day; *mfy/mfy*, 512,331.7 ± 202,719.5 cases/day, $p < 0.01$ for genotype). Note that scales for the y axis of *mfy/mfy* and control mice are shown at the right and left sides, respectively.

(C) In the elevated plus maze test, αChn-deficient mice exhibited decreased anxiety-like behavior. These mice exhibited a significant increase in the percentage of time spent in open arms compared with control (*$p < 0.05$).

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cognition. Similarly, although disruption of WAR/srGAP3, BCR, or ABR, which is a RacGAP, results in impaired memory (Carlson et al., 2011; Oh et al., 2010), the significance of the developmental roles of these molecules in adult cognition is unclear. On the contrary, in the present study, we generated and compared several types of conditional KO mice and found that enhanced learning was observed when α2-chimaerin is deleted from developmental stages, but not when it is deleted only in adulthood. These results clearly indicate that function of α2-chimaerin during development, but not that in adulthood, is indispensable for normal cognition in adulthood. Moreover, it is intriguing that hippocampus-dependent learning of DT-αChnKO and DT-α2KO mice was increased and not decreased. Thus, understanding Rho GTPase signaling is important not only in the context of the pathogenesis of developmental psychiatric disorders but also in the context of developmental mechanisms underlying normal cognition. None of the previously reported KO mouse lines for Rho GTPases and their regulators (GEFs and GAPs) exhibit learning enhancement. Furthermore, recent reports suggested that inhibition of RacGAP SRGAP2 function by its human-specific paralog, SRGAP2C, contributes to the evolution of the human neocortex (Charrier et al., 2012). Thus, RacGAPs and other Rho GTPase regulators may contribute to the adjustment of cognitive abilities not only during development but also in evolution.

What cellular abnormalities underlie the enhancement of hippocampus-dependent learning induced by the absence of developmental α2-chimaerin? Considering the biochemical features of α2-chimaerin as a RacGAP, which regulates actin cytoskeleton dynamics, morphological abnormalities in axons or dendritic spines are primary candidates. α2-chimaerin-deficient mice display abnormal axon guidance of the CST and CPG (Beg et al., 2007; Iwasato et al., 2007; Wegmeyer et al., 2007). In humans, gain-of-function mutations of the α-chimaerin gene *CHN1* cause Duane's retraction syndrome, an eye movement disorder caused by the disruption of axon guidance in the ocular motor system (Miyake et al., 2008). A recent report illustrates that β2-chimaerin, a homolog of α2-chimaerin, is involved in axonal pruning in the hippocampus (Riccomagno et al., 2012). Therefore, it is possible that in the α2-chimaerin-deficient hippocampus, axonal abnormalities exist and alter some circuit properties, such as excitatory and inhibitory balance. In contrast, to date, there are no reports of the involvement of α2-chimaerin in spine morphogenesis. However, we believe it is likely that spine morphogenesis is altered in the α2-chimaerin-deficient hippocampus because α2-chimaerin protein is enriched not only in axonal growth cones but also in dendritic spines (Iwasato et al., 2007; Shi et al., 2007). Another possibility is that neuronal migration could be altered in the α2-chimaerin-deficient hippocampus. A recent study using a knockdown approach suggests a role of α2-chimaerin in cortical neuron migration (Ip et al., 2012). However, we consider that this possibility is unlikely because we have not found abnormalities in cellular positioning in the brains of DT-α2KO mice (data not shown). Extensive studies will be required to investigate the alterations of neuronal circuits that may underlie the enhancement of hippocampus-dependent learning in α2-chimaerin-deficient mice.

α1-chimaerin and α2-chimaerin exhibit different biochemical properties and roles in vitro, as well as distinct temporal expression patterns in the mouse brain (Buttery et al., 2006; Hall et al., 2001; Lim et al., 1992; Van de Ven et al., 2005). By systematically generating and analyzing isoform-specific α-chimaerin KO mice, the present study showed that the α1 and α2 isoforms have distinct roles in brain function. We found that the α2 isoform, but not the α1 isoform, is involved in contextual fear conditioning (Figure 3). Locomotor activity is elevated in α2KO mice, whereas it is normal in α1KO mice (Figure 2; Table S1). Basal anxiety is higher in α2KO mice but lower in α1KO mice (Figure 2; Table S1). In flies, the RhoGAP18B isoforms RhoGAP18B-RC and RhoGAP18B-RA regulate distinct behavioral responses to ethanol (Rothenfluh et al., 2006). The present study provides in vivo evidence that distinct isoforms of a Rho family GAP, encoded by a single gene, play different roles in the mammalian nervous system. To determine which biochemical properties and temporal expression patterns are important for each isoform-specific function, further studies, such as knockin experiments in which α1-chimaerin is replaced with α2-chimaerin, will be required. In the current study, we systematically generated and analyzed a series of mice with global and conditional KO of α-chimaerin and its isoforms. Our results suggest that α2-chimaerin acts during development to establish normal cognitive ability in adulthood.

EXPERIMENTAL PROCEDURES

Animals

All procedures of animal care and use were approved by the institutional guidelines of NIG and BSI. αChn-flox, α1-flox, and α2-flox mice were generated by gene targeting using MS12 embryonic stem cells derived from the C57Bl/6 (B6) strain of mouse. αChnKO, α1KO, and α2KO mice were generated by expressing Cre recombinase in the germline of αChn-flox, α1-flox, and α2-flox mice, respectively. All of these mice were generated and maintained in the pure B6 genetic background. Details are described in the Supplemental Experimental Procedures.

Generation of Antibody

Keyhole limpet hemocyanin-coupled synthetic peptides (MPSKESWSGRKANRATV) corresponding to the N terminus of α1-chimaerin were used to raise a rabbit polyclonal antibody.

(D) Locomotor activity was not altered in DT-αChnKO mice (Control, 177.8 ± 10.2 m; DT-αChnKO, 193.5 ± 10.5 m; p = 0.35).
(E) DT-αChnKO mice showed normal anxiety-like behavior (open, p = 0.51; center, p = 0.54; closed, p = 0.62).
(F) Locomotor activity was not altered in α1KO mice (Control, 218.9 ± 7.0 m; α1KO, 204.9 ± 8.2 m; p = 0.20).
(G and H) α1KO mice showed increased anxiety-like behavior in both open-field and elevated plus maze tests. (G) α1KO mice displayed decreased time spent at the center zone compared with control mice in open field (*p < 0.05). (H) α1KO mice displayed increased time spent in closed arms in elevated plus maze (*p < 0.05).
(I) α2KO mice displayed higher locomotor activity (Control, 195.1 ± 9.0 m; α2KO, 482.7 ± 96.2 m; p < 0.05 for genotype).
(J) α2KO mice displayed no anxiety-like behavior. Control mice showed preference for closed arms compared to open arms (***p < 0.001, paired t test). In contrast, α2KO mice showed no preference for closed arms. The time spent at open arms was not different from that at closed arms in α2KO mice (p = 0.79, paired t test). Data are shown as mean ± SEM. NS, not significant.

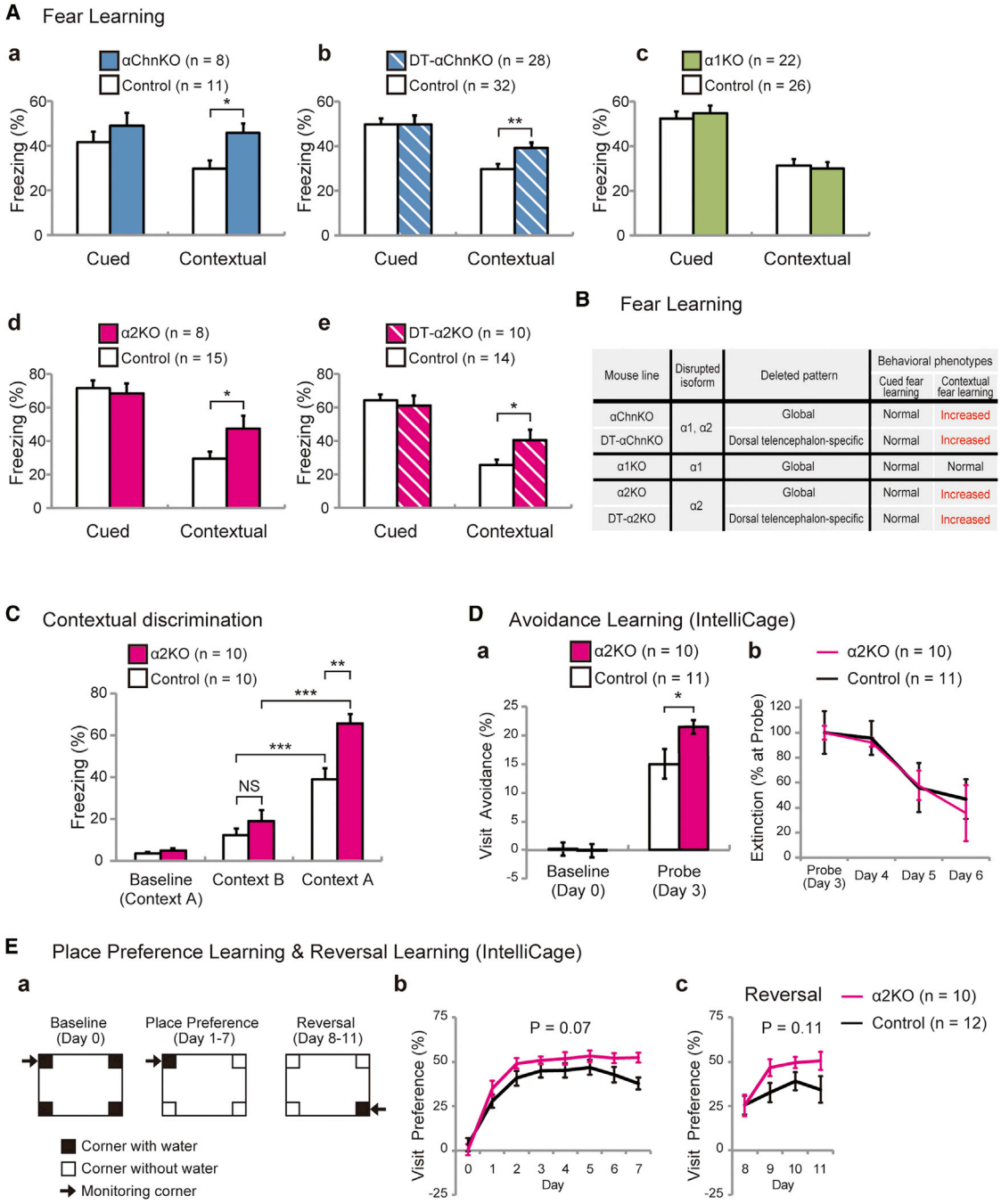

Figure 3. Isoform- and Region-Specific Roles of α-Chimaerin in Learning and Memory

(A) Contextual fear learning is increased in αChnKO (*p < 0.05; Cued, p = 0.34) (a), DT-αChnKO (**p < 0.01; Cued, p = 0.99) (b), α2KO (*p < 0.05; Cued, p = 0.70) (d), and DT-α2KO (*p < 0.05; Cued, p = 0.64) (e) mice, but not in α1KO (p = 0.79; Cued, p = 0.59) (c) mice.

(B) Summary of the representative phenotypes of the KO mouse lines in the fear-conditioning test.

(C) Both α2KO and control mice showed higher freezing in the conditioned context (Context A) than in the novel context (Context B; α2KO, ***p < 0.001; Control, ***p < 0.001, paired t test). α2KO mice exhibited enhancement of freezing compared to control mice in the conditioned context (Context A, **p < 0.01) but not in the Context A before the footshock (Baseline, p = 0.67) or in the novel context (Context B, p = 0.30).

(D) "Visit Avoidance," calculated by subtracting the visit percentage to punishment corner from the chance level (25%), was assessed using the IntelliCage system. (a) The percentage of Visit Avoidance during the baseline session and probe trial (Baseline, p = 0.84; Probe, *p < 0.05) is shown. (b) Extinction performance was quantified as the Visit Avoidance normalized to the value of the probe trial. α2KO mice exhibited normal extinction (two-way ANOVA; $F_{(1,19)} = 0.048$; p = 0.83 for genotype).

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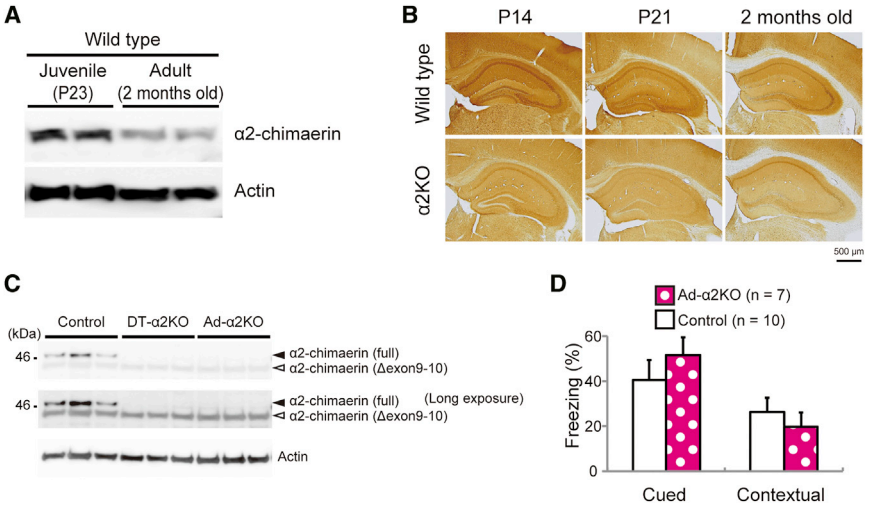

Figure 4. α2-Chimaerin in Adulthood Is Dispensable for Contextual Fear Learning

(A) Western blot analysis of WT mouse hippocampus showed higher levels of α2-chimaerin expression during development than in adulthood.

(B) Immunohistochemical analysis of the slices revealed that α2-chimaerin expression levels were high in the developing hippocampus (P14 and P21).

(C) Western blot analysis of the adult (3-month-old) hippocampus using an α2-chimaerin-specific antibody for control (*Chn1*$^{\alpha 2flox/-}$), DT-α2KO (*Emx1*$^{Cre/+}$;*Chn1*$^{\alpha 2flox/-}$), and Ad-α2KO (tamoxifen-treated *SLICK-H*;*Chn1*$^{\alpha 2flox/-}$) mice. In DT-α2KO and Ad-α2KO mice, intact α2-chimaerin protein (black arrowheads) was undetectable even with longer exposure. Truncated α2-chimaerin (Δexons 9–10: white arrowheads), which lacks RacGAP activity, is expressed from the αChnKO allele.

(D) Contextual fear learning is not increased in Ad-α2KO mice ($p = 0.50$; Cued, $p = 0.40$). Data are shown as mean ± SEM.

Behavioral Tests

IntelliCage test was carried out with 5- to 6-month-old female mice. All other behavioral tests were carried out with 2- to 6-month-old male mice. All quantitative analyses were conducted under a strict genotype-blind condition. Details are described in the Supplemental Experimental Procedures.

Human Study

All experiments were performed in Osaka University, in accordance with the World Medical Association's Declaration of Helsinki and approved by the Osaka University Research Ethics Committee. All subjects were biologically unrelated and of Japanese ethnicity. The autistic trait was assessed using the Japanese version of the autistic-spectrum quotient, and arithmetic ability was measured using the Japanese version of the Wechsler Adult Intelligence Scale, third edition. Details are described in the Supplemental Experimental Procedures.

Statistical Analyses

Unpaired two-tailed Student's t test was used unless otherwise indicated. Details are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.07.047.

AUTHOR CONTRIBUTIONS

R.I., S.I., and T.I. designed the research. R.I. performed most experiments and data analyses except for mouse generation and human studies. M.I. and T.I. generated flox and KO mouse lines. T.I. designed the α1-chimaerin-specific antibody. K.O., Y.Y., H.Y., and R.H. performed human SNP analyses. Y.K., A.M., and M.T. partly contributed to the behavioral experiments. R.I. and T.I. wrote the paper with K.O., R.H., and S.I. S.I. and T.I. supervised the project.

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(E) Place-preference and reversal-learning tests were performed, and the performance was quantified as the percentage of "Visit Preference," which is the percentage of visits to the monitoring corner subtracted by the chance level (25%). (a) Experimental scheme is shown. (b) The Visit Preference in the place-preference learning test (two-way ANOVA; $F_{(1,20)} = 3.61$; $p = 0.07$ for genotype) is shown. (c) The Visit Preference in the reversal-learning test (two-way ANOVA; $F_{(1,20)} = 2.78$; $p = 0.11$ for genotype) is shown. Data are shown as mean ± SEM.

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