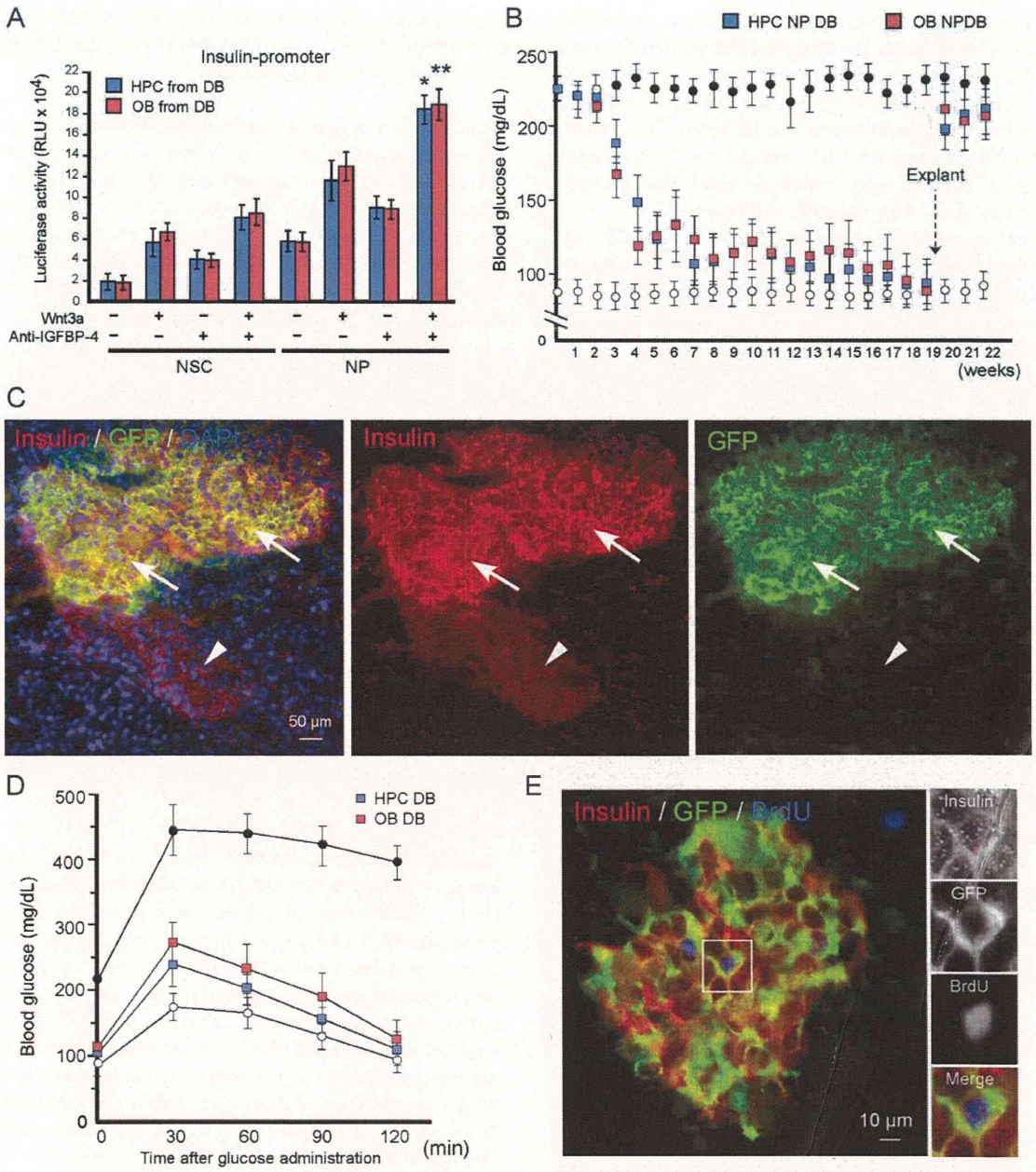


**Figure 5. Therapeutic treatment of diabetes by adult NSC transplantation.**

**A.** Insulin expressions in HPC and OB NSCs derived from DB rats. Insulin promoter activity was measured by using the luciferase reporter construct in adult HPC and OB NSC cultures that were prepared from type II DB rats [GK/slc rats, male; HPC from DB (blue bars) and OB from DB (red bars)]. The effect of Wnt3a ligand and anti-IGFBP-4 on the reporter was assessed in both undifferentiated NSCs and the NP cells. $^{*}p < 0.01$ and $^{**}p < 0.001$.

**B.** The effect of the grafted adult NPs in DB animals. HPC and OB NPs from type II diabetes rats were prepared *ex vivo* under treatment with Wnt3a and anti-IGFBP-4, and the resultant HPC NP DB and OB NP DB were transplanted into the pancreas of diabetes rats. Open circles, normal rats control; blue squares, HPC NP DB; red squares, OB NP DB; closed circles, diabetes GK/slc rats.

**C.** IHC of tracing CAG promoter driven EGFP cells (OB NP DB) in type II GK/slc DB rats. Co-localizing cells for insulin and GFP are indicated by white arrows. The preexisting islet expressing insulin at lower levels is indicated by the white arrowhead. Insulin, red; CAG-GFP, green; DAPI, blue.

**D.** Average of blood glucose levels in the response to intraperitoneal glucose tolerance test. Eighteen weeks after the implantation of adult NPs into rat pancreas, blood glucose levels were measured to glucose administration. Open circles, wild-type rats; blue squares, HPC NP DB grafts into DB rats; red squares, OB NP DB grafts into DB rats; closed circles, GK/Jcl DB rats. $n = 4$ *per* each group.

**E.** IHC of tracing HPC NP DB cells in DB rats. Cells in the white square region are magnified and shown in separate panels at right. Insulin, red; CAG-GFP (GFP), green; BrdU, blue.

  

results in decreased survival and maturation of newborn neurons (Gao et al, 2009). The diabetes-dependent impairments of adult hippocampal neurogenesis and synaptic plasticity might result from the diminished Wnt signalling that leads to *NeuroD1* down-regulation. Because *NeuroD1* is important for activating insulin mRNA expression, the decline in Wnt/β-catenin signalling in diabetes may impair *de novo* insulin mRNA expression by attenuating *NeuroD1* expression.

Our present study revealed that adult HPC and OB NSCs possess the intrinsic ability to generate insulin-producing cells via Wnt/β-catenin signalling. Since the successful isolation of NSCs from human adult OB was first reported, OB NSCs have attracted considerable attention as an accessible source of NSCs for studying various diseases. Adult HPC and OB NSCs derived from DB animals retained the ability to produce insulin in *ex vivo* culture (Fig 5). We further determined that the best source of insulin supply for transplantation into DB animals was adult NPs that had been committed into early neuronal lineage and had been treated with anti-IGFBP4 and Wnt3a to rescue their impaired Wnt signalling in DB animals. Grafted adult HPC and OB NPs reduced blood glucose levels for more than 2 months in DB rats (Fig 5B).

Various cell-based approaches have been explored for diabetes treatment: (1) differentiation to the β cell lineage from embryonic stem cells and/or induced pluripotent stem cells (iPS cells) (Lumelsky et al, 2001; Tateishi et al, 2008; Zhang et al, 2009), (2) direct conversion of differentiated cells into β cells (Thorel et al, 2010; Zhou et al, 2008) and (3) trans-differentiation of adult progenitor cells (Seaberg et al, 2004). This study provides an example of the direct use of adult stem cells from one organ to another without introducing inductive genes. Using adult NSCs for treating diabetes is potentially advantageous because donors are not required, the introduction of inductive genes is not necessary, and extracellular and endogenous regulation suitably resembles that employed by adult islet endocrine cell lineages. Although further studies, including the validation of other adult NSCs, should be forthcoming, the basic strategy outlined here should be useful for preliminary treatment of diabetes using the patient's own NSCs before the disease progresses.

## MATERIALS AND METHODS

### Cell preparation and culture

Female 7- to 8-week-old Fisher 344 rats with a body weight of 100–150 g were used (Charles River Japan, Inc). All animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the National Institute of Advanced Industrial Science and Technology. Adult NSCs were prepared and maintained as described previously (Dictus et al, 2007; Gage et al, 1995). The studies are described in more detail in the Supporting information.

### *In situ* hybridization

Brains were dissected from freshly euthanized Fisher 344 rats and put in ice-cold saline until blocking. Brains were then placed in plastic blocks in insulin OCT (TissueTek) and frozen. Sections were cut at 15 μm by cryostat (LEICA CM1850. Leica). Brain sections on the slide-glass were hybridized with labelled riboprobes as described in more detail in the Supporting information.

### Enzyme-linked immunosorbent assay of insulin

The insulin content of brain, HPC, femoral muscle, pancreatic head and pancreatic tail was measured by ELISA. Liquid nitrogen snap-frozen tissues of each age (7-week-old and 16-month-old Fisher 344 rats) were immediately homogenized and immunoreactive insulin was determined using rat insulin ELISA kit (Shibayagi, Gunma, Japan) according to the manufacturer's protocol. For details, see the Supporting information.

### DNA Microarray

Total RNA from HPC/OB was extracted using ISOGEN (NipponGene) and labelled with Cy3. Samples were hybridized with Whole Rat Genome Microarray (G4131F, Agilent). Each sample was hybridized with the one colour protocol. Arrays were scanned with a G2505C Microarray Scanner System (Agilent). Data analysed by using GeneSpring GX11.0 software (Agilent). Two normalization procedures were applied; first, signal intensities less than 1 were set to 1. Then each chip was normalized to the 75th percentile of the measurements taken from that chip. Baseline transformation of those data did not perform. Genes with 'present' and 'marginal' flag value in all samples were used for analyses (32,622 genes). GO terms enriched with *p*-value cut off of 0.1 are extracted. The raw microarray data were submitted to the Gene Expression Omnibus (GEO) microarray data archive (http://www.ncbi.nlm.nih.gov/geo/) at the NCBI (accession numbers GSE27956).

### Immunohistochemical analysis (IHC)

Immunofluorescence studies were primarily performed as described previously (Kuwabara et al, 2009): mouse monoclonal-beta tubulin-III (TUJ1; 1:500; Promega), guinea pig anti-insulin (1:300; Sigma), goat anti-C-peptide (1:250; Linco Research), goat anti-NeuroD (1:100; Santa Cruz), guinea pig anti-GFAP (1:500; Advanced Immunochemical Inc.), mouse anti-glucagon (1:300; Immuno), goat antibody to Wnt3 (1:100, Everest), mouse antibody to nestin (BD Biosciences), rat anti-BrdU (1:250; Abcam) and DAPI (Wako). All secondary antibodies were obtained from Jackson ImmunoResearch. The images were analysed using a Carl Zeiss LSM confocal imaging system (LSM 510; Carl Zeiss, Tokyo, Japan) or Olympus FV1000-D confocal microscope (Olympus Corporation, Tokyo, Japan).

### Microscopic analysis and quantification

Microscopic analysis and quantification were completed as previously described (Kuwabara et al, 2009). Slides were coded during IHC analysis, and the code was not broken until after analysis was complete. Briefly, quantification of cell number within the DG of HPC was performed using the 20X objective of a Carl Zeiss LSM confocal imaging system (LSM 510; Carl Zeiss) or Olympus FV1000-D confocal microscope (Olympus Corporation) by an observer blind to experimental groups. Wnt3+, Insulin+, GFP+, BrdU+ and AC3+ cell quantification and morphology phenotyping were completed in every 12th 40-μm coronal section throughout the SGZ and outer portion of the GCL of the DG (bregma, −0.80 to −4.20 mm). Phenotypic analysis and co-localization of GFP+ cells with various markers in the DG were

  

## The paper explained

### PROBLEM:

The number of patients receiving care for type 1 and type 2 diabetes has more than doubled since the past decade. In type 1 diabetes, the patient's immune system aberrantly destroys the insulin-producing β cells of the pancreas. Type 2 diabetes is caused by insulin resistance, progressively reaching the point where β cells can no longer produce enough additional insulin. Cell replacement therapy can be an effective strategy to treat diabetes; however, insufficient supply of β cells from human organ donors is a major issue. Using stem cells as a potential source for deriving new β cells in a safe and easy way has long been awaited for.

### RESULTS:

We demonstrated that subpopulations of neurons and adult NP cells express insulin in the brain. Adult NSCs, derived from the HPC and OB, give rise to insulin expressing cells. Adult NPs from DB rats could still generate insulin-producing cells, and the process requires the activation of NeuroD1 transcription factor via Wnt3 signalling. Under normal circumstances, insulin-expressing cells of neuronal lineage do not express phenotypic markers of pancreatic β cells. However, after transplantation into the pancreas, these neuronal cells not only increase their production of insulin but also start to express several transcription factors characteristic for pancreatic beta cells. As an approach to stem cell therapy that could be applied without gene transfer techniques, we supplied adult NPs from DB rats into the pancreases of DB rats. We found that this treatment induced insulin expression, reduced blood glucose levels and up-regulated insulin levels. Removal of these implants led to elevated levels of blood glucose, indicating that transplanting neural progenitor/stem cells into the pancreas could be a useful approach for treating diabetes.

### IMPACT:

Our data indicate that adult NSCs are a relevant and safe cell source of insulin-producing cells. OB-derived NSCs are particularly useful because of their easily accessible location and their ability to generate insulin-producing cells, as hippocampal NSCs do. The findings of this study indicate the potential value of this technique for treating human diabetes without gene transfer, and would contribute as a novel strategy to overcome donor issues in cell replacement therapy of diabetes.

done on randomly assigned coronal sections throughout the SGZ using a confocal microscope (Carl Zeiss LSM confocal imaging system; 40X objective). Carl Zeiss Confocal Software was used for scanning, optical sectioning in the Z plane and 3D rendering.

#### Chromatin immunoprecipitation (ChIP) and qRT-PCR

ChIP was performed essentially according to the manufacturer's protocol by using a commercial kit (Upstate). Briefly, DNA was cross-linked to protein with formaldehyde. Cellular lysate was obtained by scraping, followed by pulse ultrasonication to shear cellular DNA. Immunoprecipitations were performed with 1.0 μg of antibodies and bound DNAs were purified by phenol:chloroform extraction. Resultant purified DNAs were analysed using quantitative PCR as described above. For details, see the Supporting information.

#### Animals

For the IHC analysis, we used 8- to 10-week-old C57/BL6 mice, 7- to 12-week-old adult female Fisher 344 rats, 3- to 4-week old young adult Fisher 344 rats, GK/slc type II DB rats and STZ- (70 mg/kg injectable dose; Wako) induced diabetic Fisher 344 rats (Type I diabetes model; 10-week-old females). The studies are described in more detail in the Supporting information.

#### Statistics

Experiments were analysed for statistical significance using a Student's *t* test, with all error bars expressed as ± standard error of mean (s.e.m.). Values of $p < 0.05$ or $p < 0.001$ were considered significant.

### Author contributions

TK, MNK, FHG and MA conceptualized and designed the experiments; TK, MNK, YO, YI, MW and KT did the experiments; TK, MNK, YI, TS, KN and FHG analysed the data and prepared the manuscript.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

## For more information

SCRC web page:
**http://unit.aist.go.jp/scrc/cie/index_en.html**

  

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# Analyses of fear memory in *Arc/Arg*3.1-deficient mice: intact short-term memory and impaired long-term and remote memory

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## ABSTRACT

**Activity-regulated cytoskeleton-associated protein (Arc/Arg3.1) was originally identified in patients with seizures. It is densely distributed in the hippocampus and amygdala in particular. Because the expression of *Arc/Arg*3.1 is regulated by nerve inputs, it is thought to be an immediate early gene. As shown both *in vitro* and *in vivo*, *Arc/Arg*3.1 is involved in synaptic consolidation and regulates some forms of learning and memory in rats and mice [1,2]. Furthermore, a recent study suggests that Arc/Arg3.1 may play a significant role in signal transmission via AMPA-type glutamate receptors [3-5]. Therefore, we conducted a detailed analysis of fear memory in *Arc/Arg*3.1-deficient mice. As previously reported, the knockout animals exhibited impaired fear memory in both contextual and cued test situations. Although *Arc/Arg*3.1-deficient mice showed almost the same performance as wild-type littermates 4 hr after a conditioning trial, their performance was impaired in the retention test after 24 hr or longer, either with or without reconsolidation. Immunohistochemical analyses showed an abnormal density of GluR1 in the hippocampus of *Arc/Arg*3.1-deficient mice; however, an application of AMPA potentiator did not improve memory performance in the mutant mice. Memory impairment in *Arc/Arg*3.1-deficient mice is so robust that the mice provide a useful tool for developing treatments for memory impairment.**

**Keywords:** Activity-Regulated Cytoskeleton-Associated Protein (Arc/Arg3.1); Knockout (Ko) Mouse; Short-Term Memory; Long-Term Memory; Reconsolidation; Ampa Receptor

## 1. INTRODUCTION

Activity-regulated cytoskeleton-associated protein (*Arc/Arg*3.1) is encoded by an effector immediate early gene and is selectively localized in neuronal dendrites [6]. *Arc/Arg*3.1 and its encoded protein are thought to play a role in activity-dependent plasticity of dendrites [7]. *Arc/Arg*3.1 mRNA is greatly increased by long-term potentiation (LTP)-inducing electrical stimuli [8,9]; administration of psycho-stimulant drugs such as cocaine [10], amphetamines/methamphetamines [11-13], and phencyclidine [14]; insulin [15], middle cerebral artery occlusion [16], electroconvulsive shock [17,18], olfactory inputs [19], mating [20], stress [21], and other stimuli that prompt neuronal activity [22,23]. The mRNA is then rapidly delivered to the dendrites [9]. Furthermore, intense synaptic activity induces selective localization of *Arc/Arg*3.1 mRNA to activated synapses [9,24]. Therefore, Arc/Arg3.1 is believed to be related to synaptic plasticity, and thus many electrophysiological and biochemical studies have been conducted to investigate this possibility [7-9,25]. These studies have revealed that Arc/Arg3.1 may be a key molecule involved in late-phase LTP, during which long-term memories (LTM) are thought to be established.

Guzowski *et al.* [1] found that rats in which *Arc/Arg*3.1 antisense oligonucleotides were infused into the hippocampus perform relatively poorly in a water-maze probe test. This result strongly suggested that Arc/Arg3.1 plays an important role in the formation of some forms of spatial LTM. Furthermore, Plath et al. [2]

developed *Arc/Arg*3.1-deficient mice and demonstrated learning and memory impairment in these mice in the Morris water maze task, contextual and cued fear conditioning, novel object recognition, and the conditioned taste-aversion test. These results indicate that *Arc/Arg*3.1 regulates a wide range of learning and memory function. According to both behavioral and electrophysiological data, they concluded that *Arc/Arg*3.1 is essential for LTM. Although the authors concluded that Arc/Arg3.1 does not play a significant role in synaptic potentiation and early LTP, they did not show adequate behavioral evidence to conclude the role of *Arc/Arg*3.1 in the fear memory (they only showed intact short-term memory (STM) 10 min after the initial trial, and intact novel object recognition test). LTM deficiency in *Arc/Arg*3.1-deficient mice was evident about 120 min after high-frequency stimuli. Thus, the additional memory tests are necessary that should be conducted at about 120 min or later after the training and/or conditioning.

Arc/Arg3.1 may be involved in the endocytosis of AMPA-type glutamate receptors [3-5] by inducing the internalization of AMPA receptors. The morphological properties of AMPA receptors in *Arc/Arg*3.1-deficient mice were not, however, analyzed, and how AMPA receptor trafficking relates to impaired LTM remains unknown.

Ube3A regulates *Arc/Arg*3.1 degradation in synapses, and mutation of Ube3A may be a cause of autism spectrum disorders [26-28] and Angelman syndrome [29,30]. Using *Ube3A*-deficient mice, Greer et al. [31] recently reported that an increase in *Arc/Arg*3.1 expression leads to a decrease in the number of AMPA receptors. These results suggest that there may be abnormal expression of AMPA receptors in *Arc/Arg*3.1-deficient mice.

In order to elucidate the memory process of *Arc/Arg*3.1-deficient mice, in our current study, we used classical fear conditioning paradigm. First, we confirmed the LTM impairment in *Arc/Arg*3.1-deficient mice. Then, we conducted a time course analysis of the memory impairment of *Arc/Arg*3.1-deficient mice, and assessed the remote memory function of the mutant mice. Furthermore, we compared the brain structure between wild-type and *Arc/Arg*3.1-deficient mice by immunohistochemically.

## 2. RESULTS

### 2.1. Successful Generation of *Arc/Arg*3.1-Deficient Mice

We generated *Arc/Arg*3.1-deficient mice with MS12 ES cells derived from the C57BL/6 mouse strain (Supplemental information). Before conducting fear-conditioning experiments, we confirmed the intact pain sensation of *Arc/Arg*3.1-deficient mice with a tail-flick and a hot-plate paradigm in another batch of mice ($n = 10$ each). In both tests, they displayed responses indistinguishable from those of wild-type littermates (data not shown). These results indicated that *Arc/Arg*3.1-deficient mice had normal pain sensation and that they and their wild-type littermates would respond equivalently to electric foot shocks. Therefore, a classical fear-conditioning paradigm was appropriate to assess their memory function.

### 2.2. *Arc/Arg*3.1-Deficient Mice Exhibited Impairment in Both Contextual Fear and Cued fear Memory

Classical fear conditioning consists of a conditioning phase, a context test, and an auditory cued test. *Arc/Arg*3.1-deficient mice exhibited a clearly lower occurrence of freezing than did wild-type mice in the context test. The mean percentage of freezing frequency was significantly lower in *Arc/Arg*3.1-deficient mice (Mann-Whitney's U-test, U = 15, $p < 0.01$; **Figure 1A**). The subsequent auditory cue test consisted of two parts: the first half was done without a cue to assess the non-specific and/or generalized fear response to the new context, and the latter half was done with an auditory cue to assess the fear response to the cue (**Figure 1B**). The mean freezing frequency of *Arc/Arg*3.1-deficient mice was also significantly lower than that of wild-type mice under both conditions (without cue: Mann-Whitney's U-test, U = 18, $p < 0.05$; with cue: U = 0, $p < 0.001$). The identical tests were replicated five times with both male and female mice. There were no differences in impaired memory performance according to sex. Female *Arc/Arg*3.1-deficient mice did, however, display a significantly lower freezing frequency (data not

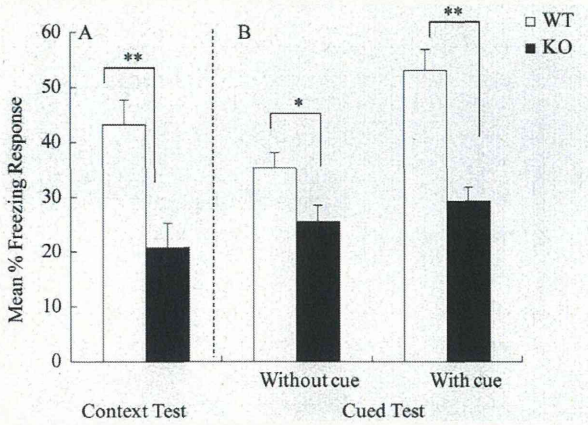


**Figure 1.** Summary of contextual and cued-fear memory performance of wild-type (WT) and *Arc/Arg*3.1-deficient (KO) mice. (A) Results of the context test. (B) Results of the cued test. Data represent the mean ± S.E.M. Asterisks indicate statistical significance (**, $p < 0.01$; *, $p < 0.05$).

 

shown). *Arc/Arg*3.1-deficient mice showed a significant decrease in the freezing response to the new context without an auditory cue. Because the results of the cued test reflect both generalized contextual fear [32] and cued fear, cued tests may be redundant. Therefore, only the contextual test trial was used in the following experiments.

### 2.3. *Arc/Arg*3.1-Deficient Mice Exhibited Intact STM but Impaired LTM

We conducted fear-conditioning tests to compare STM, LTM, and the retention of LTM in wild-type and *Arc/Arg*3.1-deficient mice. In the initial context test (4 hr after conditioning), the mean percentage of freezing of *Arc/Arg*3.1-deficient mice was indistinguishable from that of wild-type mice (Mann-Whitney's U-test, $U = 42$, n.s.; **Figure 2**). In the second context test conducted 24 hr after conditioning, the mean percentage of freezing of *Arc/Arg*3.1-deficient mice was, however, significantly lower than that of wild-type mice (Mann-Whitney's U-test, $U = 24$, $p < 0.05$; **Figure 2**). In the 1-week and 4-week tests, the mean percentage of freezing of *Arc/Arg*3.1-deficient mice was also significantly lower than that of wild-type mice (1-week test: Mann-Whitney's U-test, $U = 2$, $p < 0.01$; 4-week test: Mann-Whitney's U-test, $U = 0$, $p < 0.01$; **Figure 2**). The freezing frequency of wild-type mice increased significantly during the experiment, contrary to that of *Arc/Arg*3.1-deficient mice, whose freezing frequency decreased significantly (Two-way ANOVA with repeated measures: genotype, $F(79,1) = 58.5$, $p < 0.001$; retention time, $F(79,3) = 2.47$, $p = 0.07$, n.s.; genotype × retention time, $F(79,3) = 12.6$, $p < 0.001$; wild-type 4 hr vs. 4 week, $t = 3.33$, $p < 0.01$; *Arc/Arg*3.1-deficient mice 4 hr vs. 4 week, $t = 4.91$, $p < 0.01$).

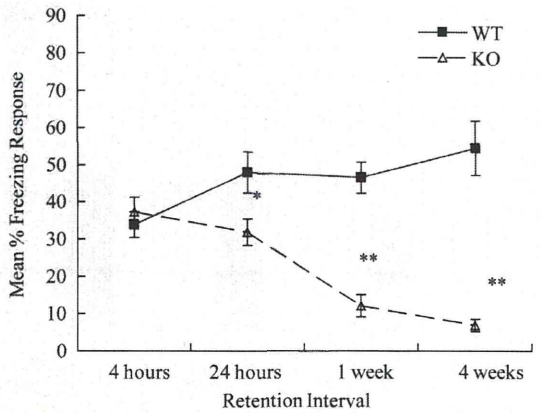


**Figure 2.** Summary of the STM and LTM tests of wild-type (WT) and Arc/Arg3.1-deficient (KO) mice. Data represent the mean ± S.E.M. Asterisks indicate statistical significance (**, $p < 0.01$; *, $p < 0.05$).

### 2.4. *Arc/Arg*3.1-Deficient Mice Showed Almost No Remote Memory of Fear

As in the STM and LTM test, the same mice were used for all experiments, because the retention curve could have been affected by the repeated exposure to the conditioning context (e.g., reconsolidation, Suzuki et al., [33]). Therefore, we conducted a remote memory test. Mice were conditioned and kept without any treatment except standard daily care for 4 weeks, and then we conducted a context test. Wild-type mice showed a high freezing frequency, but *Arc/Arg*3.1-deficient mice did not show any freezing response (Mann-Whitney's U-test: $U = 0$, $p < 0.01$; **Figure 3**).

### 2.5. AMPA Receptors Were Expressed at Higher Levels in the Hippocampus of Arc/Arg3.1-Deficient Mice

Immunohistochemical analyses were conducted to clarify the distribution of neuronal processes and synapses. Although increased immunoreactivity for neuronal processes (NF-M, MAP1A) was detected in the cerebral cortex of *Arc/Arg*3.1-deficient mice, no substantial differences were detected in the hippocampal region (**Figure 4**). Nevertheless, immunoreactivity for pre- and post-synaptic proteins (SYP, HOM) was somewhat increased in the hippocampus of *Arc/Arg*3.1-deficient mice (**Figure 4**). We also found increased immunoreactivity for GluR1 in the CA1 region and dentate gyrus (DG) of the hippocampus and for SYP in the CA3 region of *Arc/Arg*3.1- deficient mice as compared with that of wild-type mice (**Figure 5**). In order to confirm the increased GluR1 reactivity in hippocampus, we calculated

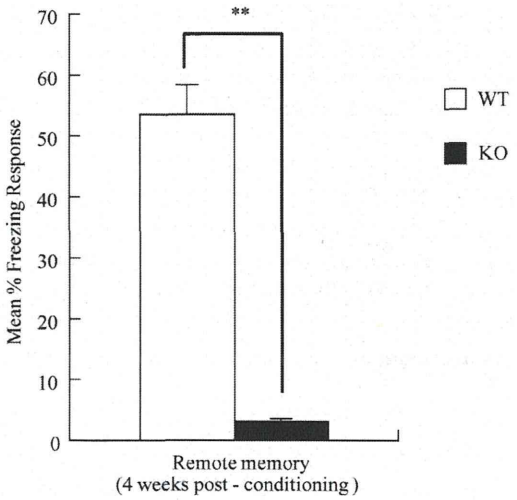


**Figure 3.** Summary of remote memory test of wild-type (WT) and *Arc/Arg*3.1-deficient (KO) mice. Data represent the mean ± S.E.M. Asterisks indicate statistical significance (**, $p < 0.01$).

 

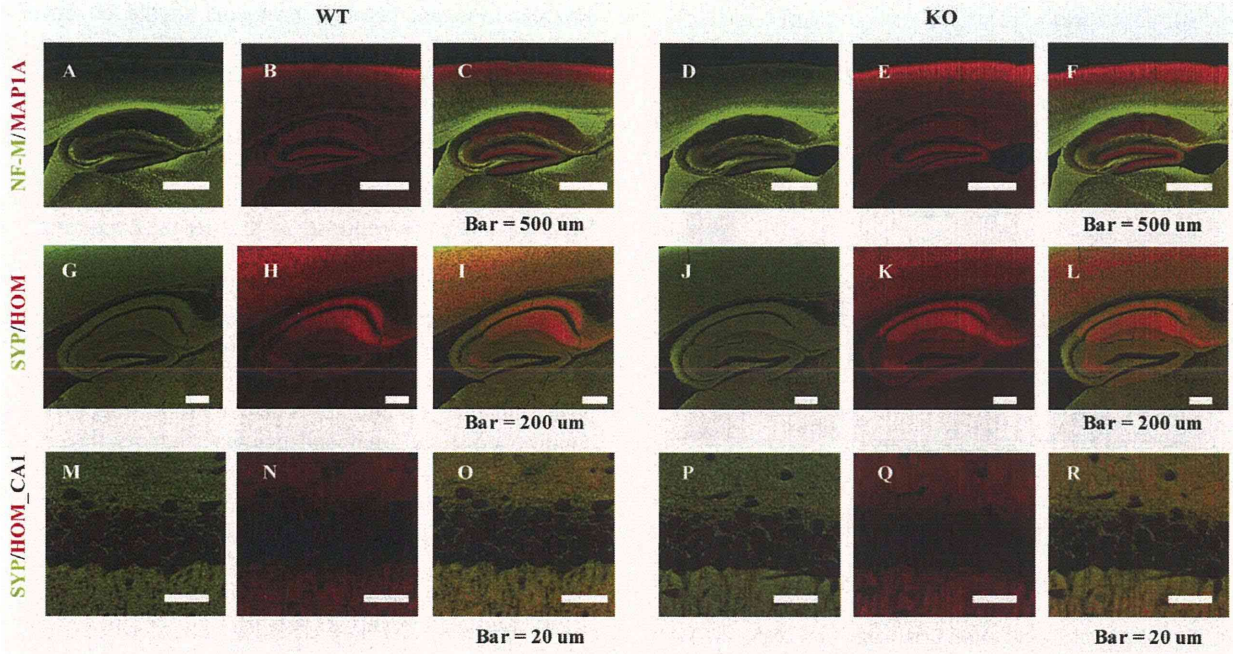


**Figure 4.** Immunohistochemical analysis of hippocampal neurons and synapses of wild-type (WT) and *Arc*/*Arg*3.1-deficient (KO) mice. A–F, Immunohistochemical images of neurofilament-m (NF-M; green) and MAP1A (red). G–R, Immunohistochemical images of synaptophysin (SYP; green) and homer (HOM; red). A–C, G–I, and M–O, wild-type mice. D–F, J–L, and P–R, *Arc*/*Arg*3.1-deficient (KO) mice. C, F, I, L, O, and R, merged images. A–F, cerebral cortex and hippocampus; scale bar = 500 μm. G–L, hippocampus; scale bar = 200 μm. M–R, CA1 region of the hippocampus; scale bar = 20 μm.

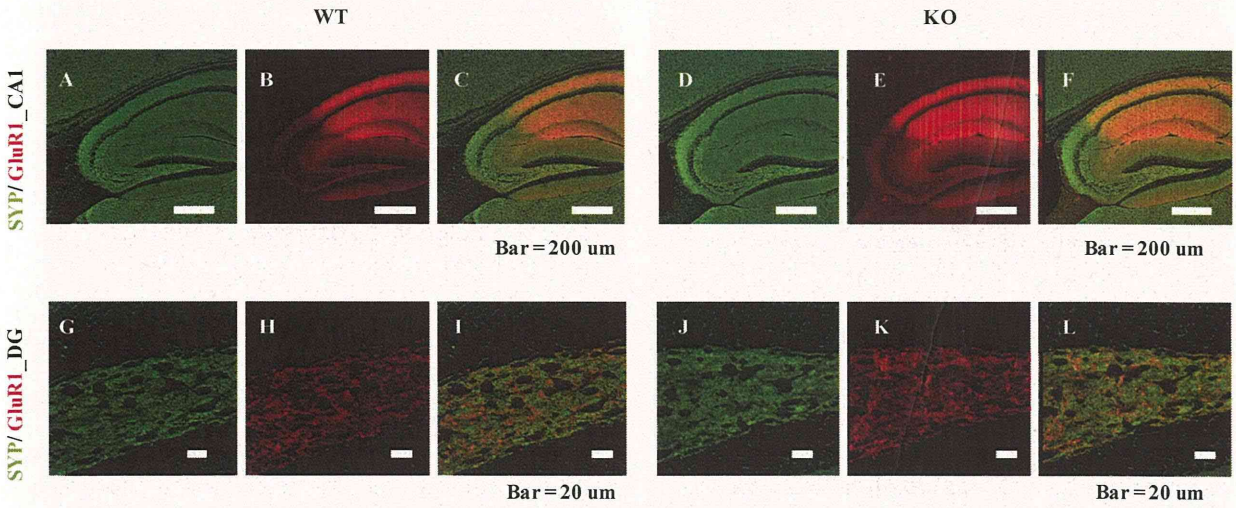


**Figure 5.** Immunohistochemical analysis of the distribution of AMPA-type glutamate receptors (GluR1) in wild-type and *Arc*/*Arg*3.1-deficient (KO) mice. Immunohistochemical images of synaptophysin (SYP, green) and AMPA-type glutamate receptors (GluR1, red). A–C and G–I, wild-type mice. D–F and J–L, Arc/Arg3.1-deficient (KO) mice. C, F, I, and L, merged images. Scale bars are identical as those of Figure 4.

the ratio of fluorescence intensity and compared between wild-type and *Arc*/*Arg*3.1-deficient mice in a semi-quantitative manner. *Arc*/*Arg*3.1-deficient mice exhibited statistically significant increase of GluR1 immunoreactivity in CA1 and CA3 (both $p<0.01$, **Figure 6**).

## 3. DISCUSSION

In our current study, we demonstrated impaired fear

 

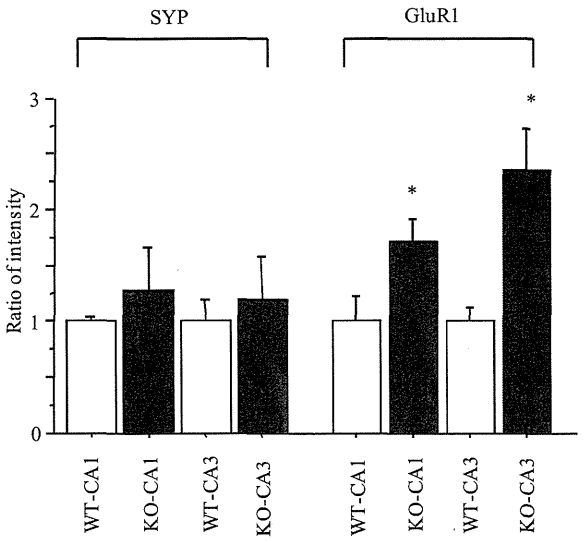


**Figure 6.** Ratio of fluorescence intensity. SYP: synaptophysin, GluR1: AMPA-type glutamate receptors, WT: wild-type mice, KO: *Arc/Arg3.1*-deficient mice. Data represent mean + SEM. *: $p<0.01$ (compared to wild-type mice).

memory in *Arc/Arg*3.1-deficient mice. In addition, immunohistochemical analysis revealed changes in synaptic structure and increased AMPA receptor expression in the hippocampus of *Arc/Arg*3.1-deficient mice. These findings suggest that *Arc/Arg*3.1 plays crucial roles not only in LTM formation but also in the memory retention process. Although an AMPA potentiator did not lead to recovery of the impaired memory in *Arc/Arg*3.1-deficient mice (Supplemental information and Supplemental **Figure 2**), this is the first study that used *Arc/Arg*3.1-deficient mice to assess the effect of drugs on memory impairment.

*Arc/Arg*3.1-deficient mice exhibited a lower percentage of freezing than did wild-type mice in the context and cued-memory test, indicating that *Arc/Arg*3.1-deficient mice were impaired in both contextual and cued memory. Many studies have reported that contextual fear reflects hippocampus-dependent memory function, and cued fear reflects amygdala-dependent memory function (for review, see LeDoux, [34]). Therefore, *Arc/Arg*3.1-deficient mice may be impaired in both hippocampus- and amygdala-dependent memory function. These results suggest that *Arc/Arg*3.1 may function in the amygdala for auditory fear memory formation as well as in the hippocampus for spatial memory formation. *Arc/Arg*3.1-deficient mice did, however, show a significant decrease in the freezing response to the new context without an auditory cue in the cued test. This result indicates that the freezing response in the cued test may reflect both generalized contextual fear and cued fear. Therefore, new experimental tasks or protocols should be developed to clarify the role of *Arc/Arg*3.1 in amygdala-dependent memory processes.

In this study, we examined STM and LTM in *Arc/Arg*3.1-deficient mice. *Arc/Arg*3.1-deficient mice exhibited unimpaired memory performance 4 hr after conditioning, indicating that their STM or early stage of LTM was intact. In contrast, 24 hr after the conditioning trial, the memory performance of *Arc/Arg*3.1-deficient mice was greatly impaired. After 4 weeks, their memory was almost completely absent, whereas that of wild-type mice slightly but significantly increased. This differential retention process may be partly due to repeated exposure to the conditioning context. Short exposure to conditioned stimuli may enhance fear memory (reconsolidation: [33,35]). Decreased fear memory by repeated exposure indicates that the reconsolidation process may also be impaired in *Arc/Arg*3.1-deficient mice. Thus, we assessed remote memory (ability to retrieve distant episodes/events) directly and confirmed complete impairment of remote memory in *Arc/Arg*3.1-deficient mice.

Immunohistochemical analyses revealed increased expression of AMPA-type glutamate receptors in hippocampal regions. This result is consistent with previous studies reporting that *Arc/Arg*3.1 may be involved in endocytosis of AMPA-type glutamate receptors and may prompt the internalization of AMPA receptors [3-5]. Although LTP, especially early-phase LTP, depends on NMDA receptors ([36] for review), activation of AMPA receptors may improve memory function in rats and mice [37,38]. Therefore, this mutant mouse strain will be useful for developing treatments including drugs for memory impairment.

## 4. EXPERIMENTAL PROCEDURES

### 4.1. The Behavioral Laboratory Environment and Housing Conditions of Mice

Mice were housed individually before transfer to the behavioral laboratory. They were kept in the laboratory during the behavioral analysis under a light/dark cycle of 12 hr/12 hr (lights on at 8:00). The laboratory was air conditioned, and the temperature and humidity were maintained at ~22 C – 23°C and 50% – 55%, respectively. Food and water were freely available except during experimentation unless otherwise indicated. We used large tweezers with soft vinyl tips to handle the mice to avoid potential differences in the handling technique of the different researchers involved in the study.

Animal experiments in this study were conducted in strict accordance with the guidelines of the Institute of Physical and Chemical Research (RIKEN) and were approved by the Animal Investigation Committee of the Institute.

 