INTRODUCTION

Information that is important to an animal’s survival is consolidated from short-term memory (STM) into long-term memory (LTM), a process that is facilitated by novelty/attention. When trivial events and momentous events occur within a short interval, the trivial experiences (STM) may be fixed to LTM. This process resembles the plasticity model of synaptic long-term potentiation (LTP) and long-term depression (LTD), leading to the hypothesized association of synaptic plasticity and memory in which activity-dependent neural plasticity is induced at appropriate synapses during memory formation.

In 1997, Frey and Morris postulated the "synaptic tagging and capture" hypothesis, which declares that LTP involves the local tagging of synapses at the moment of its induction. These tags capture plasticity-related proteins to prolong the synaptic potentiation. The hypothesis was tested initially in vitro using hippocampal slice preparations and was recently demonstrated in vivo in rats. During synaptic tagging and capture, weak activation of a synaptic population by protein synthesis-independent early-LTP/LTD sets a "synaptic tag". This tag captures the plasticity-related proteins synthesized with late-LTP/LTD activity, resulting in the consolidation of weak synapses. Tags and plasticity-related proteins may include BDNF, TrkB, CaMKII, and PKMz. Additionally, neuropsin (NP) was identified as an LTP-specific tag.

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NP is an extracellular serine protease expressed in the hippocampus and amygdala, two brain regions known for neural plasticity in the adult brain. NP is secreted and stored in the extracellular space as an inactive precursor that is transiently converted to an active form during neural activity, with roles in LTP, working memory, and anxiety.\(^\text{13-18}\)

As synaptic plasticity is one neural component of information storage,\(^\text{19,20}\) the persistence of memory should parallel the persistence of synaptic potentiation.\(^\text{21}\) Interestingly, unrelated novelty or surprise can stabilize memories, even for inconsequential events that are typically forgotten, resulting in “flashbulb memories.”\(^\text{22}\) This has been demonstrated in rats, where exploration in a novel environment (open field) around the time of inhibitory avoidance (IA) training prolongs the memory related to learning the task.\(^\text{23}\) With this approach, STM induced by a weak training paradigm is converted to LTM if animals experience a strong event in the time window around the training. Similar findings have been shown for contextual fear conditioning, spatial object recognition (SOR), and taste memory.\(^\text{24,25}\) This process, termed “behavior tagging,” relies on protein synthesis induced by the strong related experience.\(^\text{23}\) To determine whether NP, which promotes neural plasticity and synaptic tagging and capture, is also involved in behavioral tagging, we examined the performance of control and NP-deficient mice learning IA and SOR tasks. The results showed the existence of neuropsin-dependent and neuropsin-independent associative LTM.

## 2 | METHODS

### 2.1 | Subjects

NP knockout (NPKO) mice and corresponding wild-type (WT) mice (aged 12-18 weeks, male) were used in this study. NPKO mice were generated as previously described\(^\text{26}\) and backcrossed into the C57BL/6J background for at least 20 generations. Animals were housed in cages with a 12 hours light/dark cycle (lights on at 07:00) at a room temperature of 23°C and given food and water ad libitum. Animals were handled for 5 minutes for 5 consecutive days before the experiment to acclimatize the animals to experimenter handling. Behavioral tasks were performed individually in all experiments, and no mouse preformed multiple tasks. No statistical method was used to predetermine sample sizes, because the sample sizes were similar to those reported in previously published papers.\(^\text{2,27}\)

Experimental procedures were in accordance with the National Institutes of Health guidelines for the care of experimental animals and an experimental protocol approved by Institutional Animal Care and Use Committee of Maebashi Institute of Technology. The procedures minimized animal suffering, and the lowest number of animals needed to produce the required results was used.

### 2.2 | Surgery and rNP infusion

Cannulation and infusions were performed as described elsewhere.\(^\text{23}\) Cannulae were implanted bilaterally by stereotaxic surgery 1.0 mm above the dorsal hippocampus (anterior-posterior [AP] -1.6 mm, lateral ± 1.2 mm relative to bregma, and 1.2 mm ventral from dura). Only data from animals with correctly placed cannula implants were included in statistical analyses. Recombinant nontagged full-length mouse neuropsin (rNP),\(^\text{28}\) which was produced using a baculovirus expression system and purified by column chromatography, was infused (0.5 ng in 2.5 μL phosphate-buffered saline pH 7.4; flow rate, 0.5 μL /min; time, 5 minutes) into each hemisphere by using an infusion pump 5 minutes before training (Figure 1D and 1E). All infusion lines were coated with 0.1 μg/mL bovine serum albumin in phosphate-buffered saline prior to rNP infusion.

### 2.3 | IA task

IA learning is a hippocampus and amygdala-dependent task. The apparatus for this was a 25 × 25 × 35 cm (length x width x height) black plexiglass box, in which a series of stainless steel bars constituting the floor were placed on a 1 cm-high 7 cm-wide white plexiglass platform in the center of the apparatus. In the training session, mice were placed on the platform. When they put four paws on the stainless steel bars, they received two weak foot shocks by an LE10026 shocker (Panlab). After this, the animals were returned to their home cage. The animals were submitted to a test session to measure STM (15 minutes after training) (Figure 1A) or LTM (24 hours after training) (Figure 1B). Memory was measured by comparing the latency to step down from the platform in the training session to that in the test session.

### 2.4 | SOR task

Mice were trained in a hippocampus-dependent SOR task consisting of a 10 minutes exploration of two identical objects located in a familiar 40 × 40 × 40 cm cube arena with black walls and a white acrylic floor. The times spent exploring both objects in the training session were similar, resulting in an exploration ratio near 50%. The animals were submitted to a test session (5 min) to measure STM (15 minutes after training) (Figure 1F) or LTM (24 hours after training) (Figure 1G). Memory was measured by comparing the exploration time of the object in a location different from that in the training session using TopScan3.0 (CleverSys Inc).

### 2.5 | Novel field

The apparatus was a 50 cm-diameter × 39 cm-high cylindrical arena with white polyvinyl chloride walls and a white acrylic floor (for IA) or a 22 × 20 × 39 cm cube arena with brown wooden walls and floor (for SOR). Fifteen-minute exploration of the novel environment (Nov) was allowed 1 hour before training (Figure 1C, D and H).
FIGURE 1  Schematic diagram of experimental procedures. Behavioral tasks were performed individually in all experiments and no mouse preformed multiple tasks. Mice were trained in an IA task with two weak foot shocks (0.1 mA, 100 ms) with a 1 s interval, and then tested for short-term memory (STM) 15 min later (A) or long-term memory (LTM) 24 h later (B). Exploration of a novel field (Nov) for 15 min was allowed 1 h before IA training (C). Exploration of a novel field (Nov) for 15 min was allowed 1 h before IA training. Mice received an infusion of recombinant neuropsin (rNP) or vehicle (phosphate-buffered saline (PBS)) before training (D). Mice received an infusion of recombinant neuropsin (rNP) or vehicle (phosphate-buffered saline (PBS)) before training, and then tested for LTM 24 h later (E). Mice were trained in a spatial object recognition (SOR) task and then tested for STM (F) or LTM (G). Exploration of a novel field for 15 min was allowed 1 h before SOR training (H).

2.6 | Statistical analysis

All data are expressed as the means and standard errors of the means (SEMs). Statistical significance was determined as indicated by applying two-way analysis of variance (ANOVA) with Tukey-Kramer post hoc test for comparisons of multiple groups. The criterion for statistical significance was a P value of <.05.

3 | RESULTS

3.1 | NP is critical for behavioral tagging of IA memory

To assess the role of NP in memory consolidation, mice were first evaluated in the IA task. In this task, both WT and NPKO mice exhibited STM, displayed as an increased latency to step off the platform 15 minutes after training (STM: WT: training, 2.7 ± 0.42 seconds, test, 15.8 ± 4.37 seconds [n = 10]; post hoc: training vs test: *P < .05; NPKO: training, 4.92 ± 0.96 seconds, test, 16.42 ± 4.23 seconds [n = 12]; post hoc: training vs test: *P < .05, two-way ANOVA Fgenotype (1, 40) = 0.21, P = .65, Ftasks (1, 40) = 15.6, P < .0005, Fgenotype×tasks (1, 40) = 0.07, P = .8) (Figure 2).

Next, we examined LTM and Nov-LTM. In the IA task, the training was not sufficient for consolidation to LTM after 24 hours (LTM: WT: training, 2.89 ± 0.65 seconds, test, 5 ± 0.91 seconds [n = 9]; post hoc: LTM training vs test: P < .05). The criterion for statistical significance was a P value of <.05. These results suggest that NP is required for associative IA memory consolidation.
3.2 | NP is dispensable for behavioral tagging of SOR memory

Both WT and NPKO mice exhibited STM of the explored object after 15 minutes (WT: STM: training, 48.86 ± 1.05% [n = 20], test, 56.60 ± 2.61% [n = 20]; post hoc: training vs test: *P < .05; NPKO: STM: training, 48.56 ± 0.72% [n = 8], test, 60.58 ± 4.40% [n = 8]; post hoc: training vs test: *P < .05, two-way ANOVA (F_ genotype (1, 52) = 0.49, P = .49, F_tasks (1, 52) = 14.2, P < .001, and F_ genotype × tasks (1, 52) = 0.66, P = .4)) (Figure 6). Next, we examined LTM and Nov-LTM. In the SOR task, LTM 24 hours later, as the mice did not display preferential exploration of the objects (WT: LTM: training, 52.14 ± 1.97% [n = 18], test, 44.54 ± 2.12% [n = 18]; NPKO: LTM: training, 47.02 ± 2.17% [n = 12], test, 51.28 ± 2.63% [n = 12]; post hoc: P = .58, two-way ANOVA (F genotype (1, 118) = 0.3, P = .58, F_tasks (3, 118) = 16.48, P < .001, and F genotype × tasks (3, 118) = 2.77, P < .05, all data of Figure 7). However, exploration of the novel environment 1 hour before training induced the formation of LTM in WT mice (Nov-LTM: training, 52.49 ± 2.02% [n = 19], test, 60.06 ± 1.74% [n = 19]; post hoc: LTM test vs Nov-LTM test: **P < .01, Nov-LTM training vs test: *P < .05 as well as in NPKO mice (Nov-LTM: training, 50.66 ± 2.37% [n = 14]; test, 63.73 ± 2.75% [n = 14]; post hoc: LTM
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**P < .01, Nov-LTM training vs test: *P < .01**)

(Figure 7). These results suggest that NP is not needed for associative SOR memory consolidation.

4 | DISCUSSION

This study revealed that NP is not involved in the formation of STM related to IA or SOR. More importantly, we found that associative LTM consolidation related to IA and SOR occurs via NP-dependent and NP-independent mechanisms, respectively.

NPKO mice display normal spatial memory acquisition in the Morris water maze. In addition, viral infection of the hippocampus to knockdown NP impairs novel object recognition memory. Thus, NP appears to contribute to STM learning. By contrast, STM formation for IA and SOR was not affected by NP deficiency in our studies. Both of these tasks involve the hippocampus and result in the formation of STM of weak stimuli that typically do not become consolidated into LTM.

**P < .01 by Tukey-Kramer analysis after two-way ANOVA**

Figure 4 Novelty-induced LTM consolidation was restored in NPKO mice that received brief (5 min) infusions of recombinant NP (rNP; 0.2 μg/ml, 5 min, 0.5 μl/min) before training, but not vehicle (phosphate-buffered saline (PBS)). Data are means ± SEMs; **P < .01 by Tukey-Kramer analysis after two-way ANOVA**

FIGURE 5 LTM was not enhanced in NPKO mice that received brief (5 min) infusions of vehicle (phosphate-buffered saline (PBS)) or recombinant NP (rNP; 0.2 μg/ml, 5 min, 0.5 μl/min) before training. Data are means ± SEMs

Associative LTM formation describes a mechanism by which STM is transformed into LTM and has been utilized to study mechanisms of so-called behavioral tagging. For example, exposure to a novel environment may stabilize the neural representations of weak memories. A necessary condition for behavioral tagging is the synthesis of plasticity-related proteins, which can be induced in the presence of novelty or stress that activates the attention system. This process incorporates synapse tagging and capture, such that during training sessions, learning tags are set up in task-specific neurons and capture plasticity-related proteins to establish LTM. In addition to new environmental stimuli, it is also important to consider familiar environmental stimuli. In our study, the mice were not exposed to a familiar environment, which can be performed by exposing the same mice twice to a same new environmental stimulus. This will be done as part of a future study.

We previously reported that NP is involved in synaptic tag formation during LTP formation. Here, we show that NP-dependent associative LTM was formed when exposure to a novel environment preceded training for IA but not for SOR. The reason for the task specificity is not clear, but differences have been reported in the
plasticity processes within the hippocampal region. Specifically, synaptic plasticity in the hippocampus during IA learning is dominated by LTP of field excitatory postsynaptic potentials in the CA1 region, whereas SOR learning is driven by LTD at Schaffer collateral-CA1 synapses. Altogether, these findings suggest that the mechanism of NP-dependent behavioral tag formation involves LTP. Furthermore, IA learning involves both the hippocampus and the amygdala, whereas only the hippocampus is implicated in SOR learning.

The results from the present behavioral tagging studies and the findings from previous electrophysiological studies support the importance of synaptic and behavioral tag formation in regulating hippocampal-dependent synaptic plasticity and associative memory. The findings reported here demonstrate that NP is a component contributing to associative LTM formation. However, further studies are needed to determine whether NP is specific for LTP-dependent processes.

5 | ANIMAL STUDIES

Experimental procedures were all in accordance with the National Institutes of Health guidelines for the care of experimental animals, and the experimental protocol was approved by Institutional Animal Care and Use Committee of Maebashi Institute of Technology.
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CONFLICT OF INTEREST
The authors declare no conflicts of interest associated with this manuscript. The authors have no conflicts of interest directly relevant to the content of this article.

AUTHOR CONTRIBUTIONS
YS, YY, and YI designed and performed the research. YS and YY analyzed the data. YS and YI wrote the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available in the supplementary material of this article.

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