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RESEARCH REPORT

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Nasal obstruction during the growth period modulates the Wnt/β-catenin pathway and brain-derived neurotrophic factor production in association with tyrosine kinase receptor B mRNA reduction in mouse hippocampus

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Abstract

There is accumulating evidence that nasal obstruction induces high-level brain dysfunction, including memory and learning deficits. We previously demonstrated that unilateral nasal obstruction (UNO) during the growth period increases the expression of brain-derived neurotrophic factor (BDNF). The expression of BDNF is regulated by the Wnt/β-Catenin pathway, which is linked to neuronal differentiation, proliferation, and maintenance. However, little is known about whether Wnt3a protein expression could be an index for modulations analyses in the Wnt/β-Catenin pathway caused by UNO during the growth period. This study aimed to investigate the effects of UNO during the growth period on the Wnt/β-Catenin pathway in the hippocampus using combined behavioural, biochemical, and histological approaches. Male BALB/C mice were randomly divided into the control (CONT; $n = 6$) and experimental (UNO; $n = 6$) groups. Blood oxygen saturation (SpO_2) levels were measured, and a passive avoidance test was performed in mice aged 15 weeks. Brain tissues were subjected to immunohistochemistry, real-time reverse transcription-polymerase chain reaction, and western blot analysis. Compared with control mice, UNO mice had lower SpO_2 levels and exhibited memory/learning impairments during behavioural testing. Moreover, Wnt3a protein, BDNF mRNA, and tyrosine kinase receptor B (TrkB) mRNA expression levels were significantly lower in the hippocampus in the UNO group than in the CONT group. Our findings suggested that UNO during the growth period appeared to modulate the hippocampal Wnt/β-catenin pathway and BDNF production in association with TrkB mRNA reduction, thereby resulting in memory and learning impairments.

Abbreviations: BDNF, brain-derived neurotrophic factor; DG, dentate gyrus; Dkk-1, Dickkopf-related protein 1; IH, intermittent hypoxia; MAPK, mitogen-activated protein kinase; RT-PCR, real-time polymerase chain reaction; SpO_2, blood oxygen saturation; TrkB, tyrosine kinase receptor B; UNO, unilateral nasal obstruction.

KEYWORDS
behavioural tests, Ki67, memory function, SpO_2, tyrosine kinase receptor B, Wnt3a

1 | INTRODUCTION

Nasal respiratory disorders have been reported to affect bodywide function through consequential disorders such as cephalalgia, feebleness, sleep disorders, and hypersomnolence (Bhattacharyya, 2015; Camelo-Nunes & Solé, 2010). During the pre-adolescent and adolescent periods, mouth breathing causes a range of craniofacial and occlusal problems, including an open bite, maxillary protrusion, and lateral crossbite (Harari et al., 2010). In recent years, the prevalence of nasal breathing disorders has increased in the younger population. It has been reported that the number of patients with allergic rhinitis increases during the growth period, and 22.5% of children aged 5–9 years and 36.6% of teenagers have allergic rhinitis (Okubo et al., 2017).

Biochemical animal studies have suggested an association between nasal obstruction and peripheral structural and functional changes, including developmental disruption of the jaw-opening reflex and aberrant tongue protrusive forces (Funaki et al., 2014; Uchima Koecklin et al., 2015). Moreover, clinical studies have demonstrated that nasal obstruction induces high-level brain dysfunction, including memory/learning deficits, and children who breathe through the nose exhibit better reading comprehension, arithmetic skills, and working memory function than those who breathe through the mouth (Kuroishi et al., 2015). In addition, patients with seasonal allergic rhinitis show lower scores in verbal learning and memory tests than healthy non-allergic controls (Trikojat et al., 2017). These findings suggest that nasal obstruction causes cognitive decline and memory learning disabilities (Ogawa et al., 2018).

The hippocampus is an essential region for memory and learning, and information from the peripheral sensory receptors is preserved temporarily and then accumulated as memory. Patients with hippocampal damage show notable defects in memory function (Eichenbaum, 2015; Zola-Morgan et al., 1986) and brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, plays an important role in the development of memory and learning (Thoenen, 2000). BDNF has a high affinity for tyrosine kinase receptor B (TrkB), and a combination of BDNF and TrkB contributes to the development of neurons, synaptic functions, memory, and learning ability (Thoenen, 1995). BDNF/TrkB signalling subsequently activates the mitogen-activated protein kinase (MAPK) pathway (Reichardt, 2006), which is necessary for memory and learning (Sweatt, 2001). Activation of the MAPK pathway is caused by the phosphorylation of p44/42 MAPK. Therefore, the level of phosphorylated (phospho)-p44/42 MAPK can be used as an indicator of BDNF/TrkB signalling activity (Alonso et al., 2002).

A previous study using growing mice with unilateral nasal obstruction (UNO) showed that the BDNF protein expression level in the UNO group was significantly increased compared with that in the control (CONT) group, and expression levels of the TrkB protein and phosphorylated-p44/42 MAPK were significantly decreased (Ogawa et al., 2018). Furthermore, the number of hippocampal neurons was reduced and memory learning function decreased in the UNO group (Ogawa et al., 2018). However, the mechanisms underlying changes in protein expression of BDNF and TrkB in the UNO group have not been clarified, and little is known about the factors contributing to changes in BDNF/TrkB signalling. Previous studies have shown that the production of the BDNF receptor TrkB is reduced in the intermittent hypoxia (IH) model (Das et al., 2018), but no studies have investigated the reduction in SpO_2 and TrkB expression associated with UNO. We investigated whether there was a link between SpO_2 reduction and TrkB production due to UNO during the growth period.

The Wnt/β-Catenin pathway is responsible for the upstream mechanism of BDNF production (Yi et al., 2012), is involved in the regulation of gene expression, and plays a role in cellular proliferation and maintenance (Grigoryan et al., 2008). In addition, Wnt signalling is involved in hippocampal neurogenesis (Marzo et al., 2016). Wnt3a, a Wnt family protein, binds to the Frizzled receptor, and β-catenin then moves into the nucleus and binds to transcription factors (Clevers & Nusse, 2012). The transcription factor, which is combined with β-catenin, binds to the DNA target region and induces the transcription of BDNF mRNA, thereby resulting in the production of the BDNF protein. When Dickkopf-related protein 1 (Dkk-1), an antagonist of Wnt3a, binds to the Frizzled receptor, β-catenin does not translocate into the nucleus, thus preventing DNA transcription (Niehrs, 2006). We decided to reveal the cause of BDNF protein increase in the UNO model by examining changes in hippocampal Wnt3a, Dkk-1, and β-catenin protein levels.

A previous study measured the number of hippocampal neurons and demonstrated that Ki67 immunostaining

could be used to evaluate the proliferative capacity of hippocampal nerves (Vinuesa et al., 2019). However, no studies have evaluated hippocampal nerve proliferative capacity in growing UNO mice. Although BDNF production in UNO has been reported during the growth period, little is known regarding whether the expression of the Wnt3a protein could be used as an index to analyse modulations in the Wnt/β-Catenin pathway caused by UNO during the growth period. In the present study, we aimed to investigate whether UNO during the growth period influences the Wnt/β-Catenin pathway in the hippocampus using combined behavioural, biochemical, and histological approaches.

2 | MATERIALS AND METHODS

All experiments described herein were approved by the Institutional Animal Care and Use Committee (protocols: A2020038 and A2020015A) and performed in accordance with the Animal Care Standards of Tokyo Medical and Dental University and ARRIVE guidelines.

2.1 | Animals

Six-day-old male BALB/C mice were obtained from Sankyo Laboratory Service (Tokyo, Japan) and randomly divided into CONT ($n = 6$) and UNO ($n = 6$) groups. Male mice were used in the study to avoid the different effects of the hormonal cycle and behavioural characteristics. Mice were housed in groups (three to four per cage) and provided with standard mouse chow and water ad libitum, under a 12-hr light/dark cycle with controlled temperature ($24 \pm 1°C$) and humidity (50%). All cages were cleaned once a week and monitored for aggressive behaviour before and during testing.

A priori power analysis was performed to determine the sample size. At 8 days of age, all mouse pups were anaesthetized through hypothermia (10 min at −18°C), and those in the UNO group underwent left-sided nasal obstruction through selective cauterization of the left external nostril, which is the simplest and most common procedure used to induce nasal obstruction in neonatal animals (Padzys et al., 2011). In particular, the tissue surrounding the left external nostril was burned by placing a surgical cauterizing instrument (1 mm in diameter) on the nostril to occlude the nostril orifice without causing mechanical or chemical damage to the olfactory mucosa (Funaki et al., 2014). After cauterization, the nostril was coated with 3% chlortetracycline (Aureomycin Ointment; Pola Pharma, Tokyo, Japan) to prevent infection. In this study, 8-day-old mice were treated with unilateral nasal congestion, because previous reports indicate that mice suffered respiratory failure and died with bilateral nasal congestion (Erkan et al., 1994). The pups were kept warm (37°C) for 30 min and then returned to their dams. Mice in the CONT group underwent a sham operation in which the cauterizing instrument was placed approximately 1–2 mm above the left nostril. Following surgery and before sacrifice, blood oxygen saturation levels and behavioural tests were evaluated by an investigator who was blinded to the groups to which the animal belonged.

2.2 | Blood oxygen saturation levels

In isoflurane-anaesthetized mice, blood oxygen saturation (SpO_2) was recorded using a pulse oximeter (MouseOx Plus; STARR Life Sciences Corp., Oakmont, PA, USA) and collar clip sensors. The sensors were placed dorsolaterally, with tips situated near the carotid arteries. SpO_2 signals were sampled at 1 Hz and averaged over 40–50 s of data near the end of each exposure (Bavis et al., 2014).

2.3 | Behavioural tests

We used the Y-maze and passive avoidance tests to evaluate memory and learning abilities in 15-week-old mice. The Y-maze apparatus comprises three arms ($300 \times 60 \times 150$ mm) that are separated by 120-degree angles and randomly assigned designations of "A," "B," and "C." The mice were allowed to freely explore the three arms for 8 min. The number of arm entries was recorded to calculate the percentage of alternations in the maze. Entry into an arm was recorded as the placement of all four limbs within the arm (Holcomb et al., 1999). The percent alternation was calculated as the ratio of the actual-to-possible alternations (defined as the total number of arm entries minus two) multiplied by 100, as shown in the following equation:

$$\textit{Percent alternation}\,(\%) = [(\textit{number of alternations}) / (\textit{total arm entries} - 2)] \times 100.$$

The passive avoidance test apparatus comprises an illuminated compartment ($100 \times 100 \times 145$ mm) and a dark compartment ($180 \times 18 \times 145$ mm) with a grid on the floor, and the two compartments are separated by a guillotine door. On the first day, the mice were habituated in the illuminated and dark compartments for 300 s. On the second day, the mice were placed into the light compartment with the guillotine door open, and we

measured the time required to enter the dark compartment, where the mice received an electrical stimulation (0.3 mA, 5 s). The time limit to enter the dark compartment was set at 300 s. Twenty-four hours later, the time to enter the dark compartment was measured again and compared with the previously recorded time to evaluate the memory and learning abilities of the mice (Yamagata et al., 2009).

2.4 | Sample collection

After the passive avoidance test, the animals were perfused with phosphate-buffered saline under isoflurane anaesthesia. The brains were dissected and divided into right and left hemispheres. The right hemispheres were separated into the cerebral cortex and hippocampus, which were frozen immediately and stored at −80°C (Ogawa et al., 2018).

2.5 | Western blotting analysis

Protein levels of Wnt3a, Dkk-1, β-catenin, BDNF, and TrkB were measured in the right hippocampal samples using western blotting (Ogawa et al., 2018). The wet weight of each hippocampus was measured, and the tissue was homogenized in 20 volumes of 20-mM Tris buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and 5-mM ethylenediaminetetraacetic acid in a handheld homogenizer (Microtec, Chiba, Japan) on ice. The samples were then centrifuged at 10,000×*g* for 20 min at 4°C. A 10-μl aliquot of the supernatant was removed and used to determine the total protein concentration using the Micro BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA). One-tenth volume of trichloroacetic acid was added to the remaining supernatant to precipitate the proteins. The samples were incubated on ice for 30 min and centrifuged at 15,000×*g* for 30 min at 4°C. The sample buffer was then added to the protein precipitate (20 μl of sample buffer/40 μg of protein), and the samples were frozen at −80°C until analysis. The samples were separated on 15% (Dkk-1) or 10% (Wnt3a, β-Catenin, BDNF, TrkB) sodium dodecyl sulfate-polyacrylamide gels at 20 mA for 45 min. The separated proteins were transferred to polyvinylidene fluoride membranes using transfer buffer (25-mM Tris, 192-mM glycine, 10% [v/v methanol]) at 200 mA for 45 min. The membranes were then blocked for 1 h at 24°C in 0.05% Tris-buffered saline-Tween 20 (TBS-T; 50-mM Tris, pH 7.4, 133-mM NaCl) containing 2.5% (w/v) skim milk powder. The membranes were washed with TBS-T and were then incubated overnight at 4°C with rabbit anti-Wnt3a polyclonal antibody (Wnt3a, AB19925, 1:1000 dilution; Abcam, Cambridge, UK, RRID:AB_778930), rabbit anti-Dkk-1 monoclonal antibody (sc-374574, 1:2000; Santa Cruz Biotechnology, Dallas, TX, USA, RRID: AB_10989416), rabbit anti-Catenin, beta polyclonal antibody (AB6302, 1:1000; Abcam, Cambridge, UK, RRID: AB_305407), rabbit anti-BDNF polyclonal antibody (BDNF, AB108319, 1:1000 dilution; Abcam, Cambridge, UK, RRID:AB_10862052), and rabbit anti-TrkB polyclonal antibody (TrkB, AB18987, 1:1000 dilution; Abcam, Cambridge, UK, RRID:AB_444716). Antibodies specific for Rabbit anti-tubulin, alpha polyclonal antibody (2144S, 1:1000 dilution; Cell Signaling Technology, USA, RRID:AB_2210548) was used as loading controls. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (7074S, 1:200,000 dilution; Cell Signaling Technology, USA, RRID:AB_2099233) diluted in TBS-T with 2.5% skimmed milk for 1 h at 24°C. For target protein detection, an ECL Prime Western Blotting Detection system (GE Healthcare, Tokyo, Japan) was used to visualize the protein bands, and ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to measure the integrated optical densities of the bands.

2.6 | Real-time polymerase chain reaction (RT-PCR)

BDNF and TrkB mRNA expression in the hippocampus and hypothalamic areas was analysed by RT-PCR. The hippocampus was removed, quickly frozen in liquid nitrogen, and stored at −80°C. Total RNA was isolated from tissues using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The total RNA concentration was corrected to 100 ng/μl using the Q5000 spectrophotometer (Tomy, Tokyo, Japan). First-strand cDNA synthesis was carried out using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed using a Stratagene Mx3000P QPCR system (Agilent Technologies, La Jolla, CA, USA). The sequences of the primers used in this analysis were as follows: forward, 5′-CGA CGACATCACTGGCTGACA-3 and reverse, 5′-CCAA AGGCACTTGACTGCTGAG-3′ for BDNF; forward, 5′-CAAGAACGAGTATGGGAAG GATGAG-3′ and reverse, 5′-TTGGCGTGGTCCAG TCTTCATA-3′ for TrkB; and forward, 5′-GTAGACAAA ATGGTGAAGG TCGGT-3′ and reverse, 5′-ACAATCTCC ACTTTGC CACTGC-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The PCR amplification was performed at 40 cycles of 95°C for

10 s and 60°C for 30 s, followed by 95°C for 10 min. The results were analysed following the $2^{-\Delta\Delta Ct}$ method with GAPDH as the internal control (Hsiao et al., 2014).

2.7 | Immunohistochemistry

On completion of the behavioural testing, the animals were perfused with phosphate-buffered saline under isoflurane anaesthesia. Brains were postfixed for 24 h in 4% paraformaldehyde, transferred to 30% sucrose until full penetration of cryoprotectant occurred, snap frozen in liquid nitrogen, and stored at −80°C. Coronal sections through the DG were collected onto slides at 40 μm thickness in a 1:6 series. The sections were selected from −1.8 mm to −5.20 mm relative to Bregma. Proliferation in the subgranular zone of the DG was determined by Ki67 immunolabelling. After blocking nonspecific antigenic sites, sections were incubated overnight at 4°C with Ki67 antibodies (AB15580, 1:1000 dilution; Abcam, Cambridge, UK, RRID:AB_443209). Sections were then incubated with biotinylated secondary antibodies (BA-1000, 1:1000 dilution; Vector Laboratories, USA, RRID:AB_2313606), followed by processing with ABC kit (Vector Laboratories) and development with 2-mM diaminobenzidine (Sigma, USA) and 0.5-mM H_2O_2 in 0.1-M Tris buffer. The sections were placed on gelatin-coated slides, air-dried overnight, dehydrated in graded solutions of ethanol, cleared in xylene, and mounted with Canada balsam. For all immunostaining analyses, at least four brains per experimental group were used. Ki67 immunostaining allows the assessment of proliferation ability in the dentate gyrus (DG) regions of the hippocampus (Yan et al., 2013). The number of Ki67+ cells was quantified under a 20× objective using the Nikon E200 microscope. For cell counting, we selected the same location and arrangement of cells in the DG regions, counted the number of neuronal cells present in each area, and calculated the cell number/40,000 μm^2. We used one section per animal for cell-counting analyses, which were performed by a single-blinded examiner who counted each section three times (Vinuesa et al., 2019).

2.8 | Statistical analysis

Statistical analyses have been carried out blindly. Quantitative results are expressed as mean ± standard deviation or by individual dot plots, as appropriate. Intergroup comparisons of data were made using the Wilcoxon signed-rank test and Mann–Whitney *U* test. The correlation analyses were performed using Pearson's correlation. SPSS software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. In all tests, $p < 0.05$ were considered statically different.

3 | RESULTS

3.1 | SpO_2 levels

We observed significant reductions in SpO_2 levels in the UNO group compared with the CONT group at 15 weeks of age. The SpO_2 level at 15 weeks of age was significantly lower in the UNO group than in the CONT group (95.1 ± 1.2% vs. 98.5 ± 0.5%; Figure 1). This finding is in line with that in a previous study, which reported a decrease in SpO_2 levels in intermittent hypoxia animals compared with those in CONT animals (Newhouse et al., 2017).

3.2 | Y-maze test

We used the Y-maze test to evaluate memory and learning abilities in 15-week-old mice. The number of arm entries and triads were recorded to calculate the percentage of alternations in the maze. The percentage of alternations was significantly lower in the UNO group than in the CONT group (50.1 ± 12.5% vs. 64.4 ± 3.3%, $p < 0.05$; Figure 2).

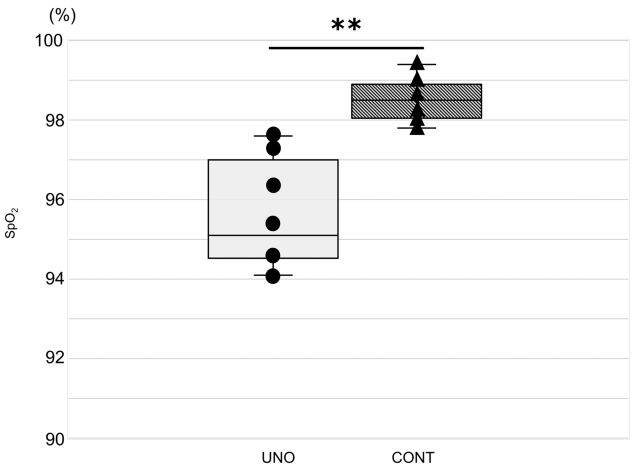

FIGURE 1 Mean SpO_2 levels at 15 weeks of age. The SpO_2 levels at 15 weeks of age were significantly lower in the UNO group (95.10 ± 1.24%) than in the CONT group (98.50 ± 0.48%). Box edges represent the upper and lower quantiles, with the median values shown by the middle line in each box. The whiskers represent the maxima and minima. *$p < 0.05$. Box edges represent the upper and lower quantiles with the median values shown by the middle line in each box. The whiskers represent the maxima and minima. Abbreviations: CONT, control group; SpO_2, blood oxygenation saturation; UNO, unilateral nasal obstruction group

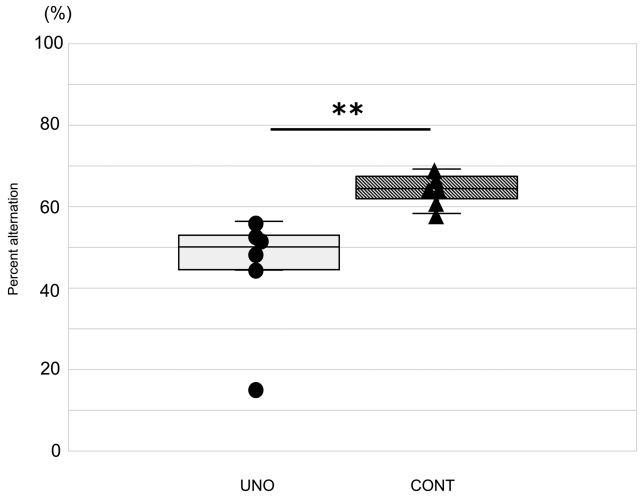

FIGURE 2 Mean percentage of alternations in the Y-maze test. The percentage of alternations in the UNO group (50.1 ± 12.50%) was significantly lower than that in the CONT group (64.4 ± 3.3%). Box edges represent the upper and lower quantiles with the median values shown by the middle line in each box. The whiskers represent the maxima and minima. *$p < 0.05$. Box edges represent the upper and lower quantiles with the median values shown by the middle line in each box. The whiskers represent the maxima and minima. Abbreviations: CONT, control group; UNO, unilateral nasal obstruction group

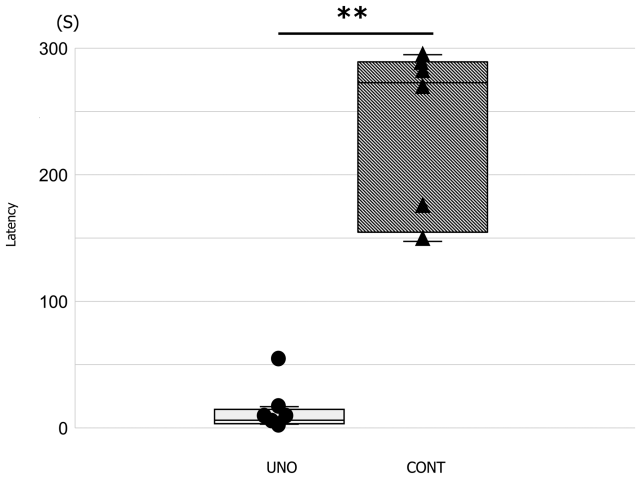

FIGURE 3 Latency in the passive avoidance test in the UNO and CONT groups. The latency in the 24-h test was significantly longer in the CONT group than in the UNO group (6.15 ± 16.34 vs. 272.85 ± 62.10). *$p < 0.05$. Box edges represent the upper and lower quantiles with the median values shown by the middle line in each box. The whiskers represent the maxima and minima. Abbreviations: CONT, control group; UNO, unilateral nasal obstruction group

3.3 | Passive avoidance test

A passive avoidance test was performed to analyse the effect of UNO on memory function. The latency in the 24-h test in the CONT group was significantly longer than that in the UNO group (272.9 ± 62.1 s vs. 6.2 ± 16.3 s, $p < 0.05$; Figure 3). These findings indicated that memory was impaired in the UNO group.

3.4 | Western blotting

Because the Wnt signalling pathway plays a critical role in BDNF production, we further analysed Wnt signalling pathway proteins in the hippocampus in the UNO and CONT groups using western blotting. As shown in Figure 4a, Wnt3a protein levels were significantly lower in the UNO group (0.65 ± 0.20) than in the CONT group (0.99 ± 0.19) ($p < 0.05$; Figure 4a). However, Dkk-1 protein levels showed no significant difference between the UNO and CONT groups (1.12 ± 0.27 vs. 0.87 ± 0.18; Figure 4b). In addition, we also evaluated the protein expression levels of β-Catenin, a downstream protein of the Wnt signalling, using western blotting analysis. Hippocampal β-catenin levels were significantly lower in the UNO group (0.73 ± 0.14) than in the CONT group (1.19 ± 0.20) ($p < 0.05$; Figure 4c). To confirm the findings of a previous study (Ogawa et al., 2018), we assessed hippocampal BDNF protein and TrkB protein expression. Hippocampal BDNF levels were significantly higher in the UNO (0.93 ± 0.15) than the CONT group (0.53 ± 0.10) ($p < 0.05$; Figure 4d). Hippocampal TrkB levels were significantly lower in the UNO (0.50 ± 0.11) than the CONT group (0.99 ± 0.18) ($p < 0.05$; Figure 4e).

3.5 | RT-PCR

BDNF and TrkB mRNA expression levels in the hippocampus are shown in Figure 5. Hippocampal TrkB and BDNF mRNA levels were significantly lower in the UNO group than in the CONT group (TrkB: 0.60 ± 0.12 vs. 0.95 ± 0.06; BDNF: 0.55 ± 0.09 vs. 0.79 ± 0.13; $p < 0.05$; Figure 5a,b). Significant effects of UNO on the production of TrkB and BDNF were observed.

3.6 | Immunohistochemistry

We evaluated the number of Ki67-positive cells in the DG regions of the hippocampus using immunohistochemical analysis. We counted the number of neurons in the DG area surrounded by a square (40.000 μm^2). The number of Ki67-positive neurons in the DG region of the hippocampus in the UNO group was significantly lower than that in the CONT group (26.44 ± 7.08 vs. 60.55 ± 7.73) ($p < 0.05$; Figure 6).

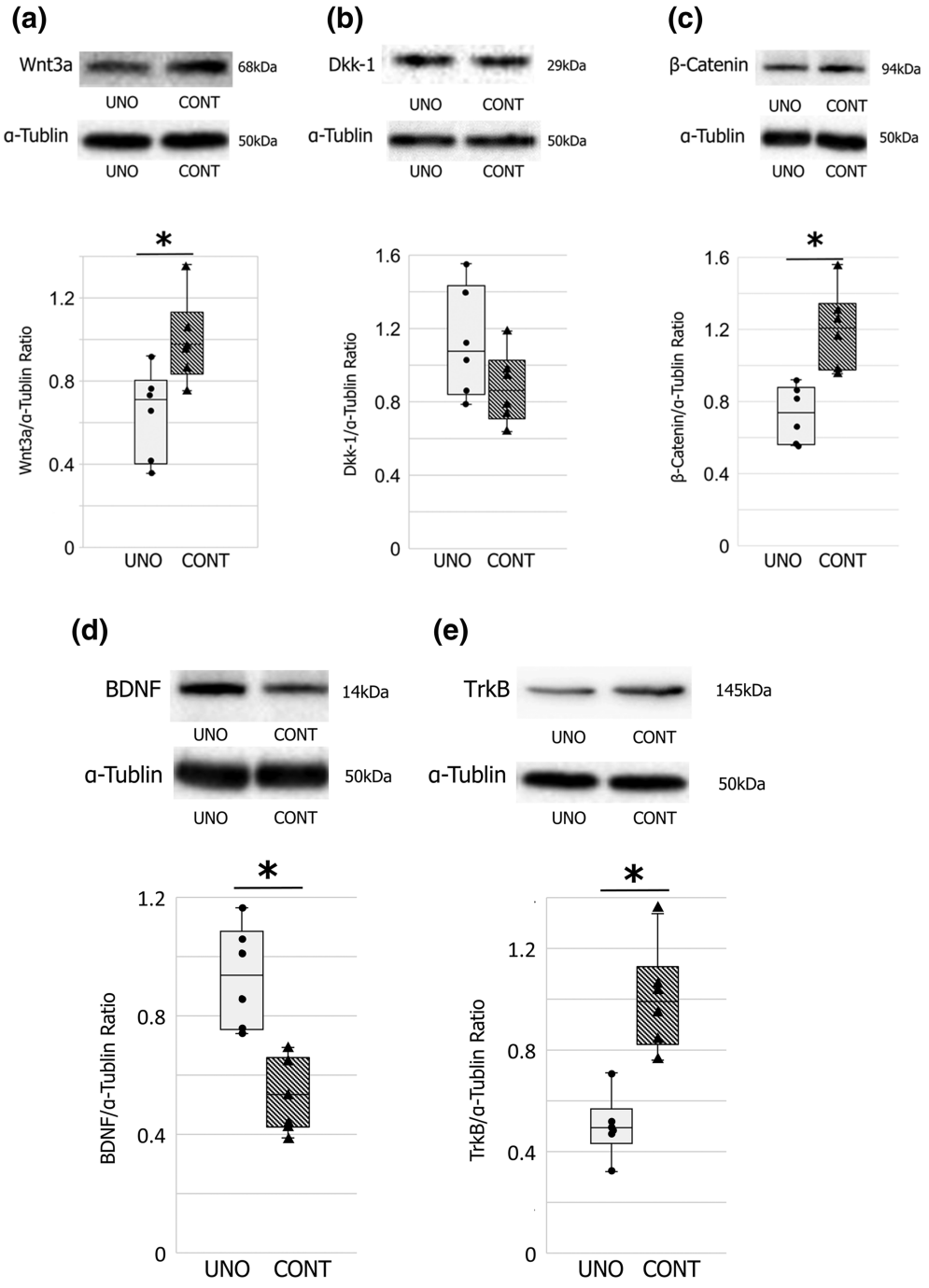

FIGURE 4 Protein levels of Wnt3a, Dkk-1, β-catenin, BDNF and TrkB in the hippocampus. Hippocampal levels of Wnt3a were significantly lower in the UNO group than in the CONT group (0.65 ± 0.20 vs. 0.99 ± 0.19) (a). Dkk-1 protein levels showed no significant difference between the UNO and CONT groups (1.12 ± 0.27 vs. 0.87 ± 0.18) (b). Hippocampal levels of β-Catenin were significantly lower in the UNO group than in the CONT group (0.73 ± 0.14 vs. 1.19 ± 0.20) (c). Hippocampal levels of BDNF were significantly higher in the UNO group than in the CONT group (0.93 ± 0.15 vs. 0.53 ± 0.10) (d). TrkB protein levels were significantly lower in the UNO group than in the CONT group (0.50 ± 0.11 vs. 0.99 ± 0.18) (e). Box edges represent the upper and lower quantiles, with the median values shown by the middle line in each box. Whiskers represent the maxima and minima. *$p < 0.05$. Box edges represent the upper and lower quantiles with the median values shown by the middle line in each box. The whiskers represent the maxima and minima. Abbreviations: CONT, control group; UNO, unilateral nasal obstruction group

3.7 | Correlation of SpO_2 levels and TrkB mRNA expression and protein levels of TrkB

A correlation analysis showed a significant positive correlation between SpO_2 levels and TrkB mRNA expression in the UNO group ($p = 0.017$, $r = 0.774$, $p < 0.05$; Figure 7a), while there was no significant correlation in the CONT group ($p = 0.475$, $r = -0.292$; Figure 7b). Moreover, there was a significant positive correlation between SpO_2 levels and protein levels of TrkB in the UNO group ($p = 0.036$, $r = 0.645$, $p < 0.05$; Figure 7c), while there was no significant correlation in the CONT group ($p = 0.960$, $r = 0.020$; Figure 7d).

4 | DISCUSSION

In this study, we investigated the effects of UNO on memory and learning function in male BALB/C mice during the growth period, with a special focus on the Wnt/β-Catenin pathway in the hippocampus and SpO_2.

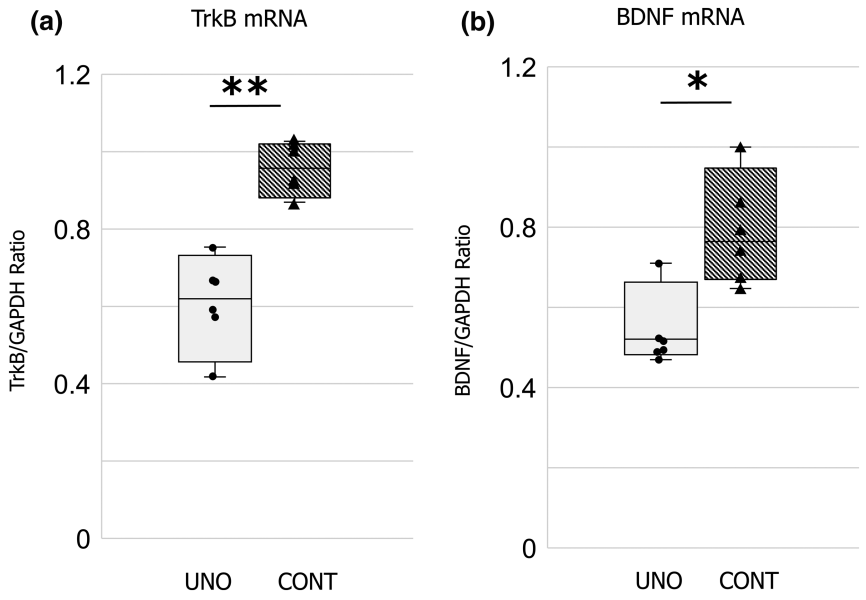

FIGURE 5 mRNA levels of TrkB and BDNF in the hippocampus. In the hippocampus, TrkB mRNA levels were significantly lower in the UNO group than in the CONT group (0.60 ± 0.12 vs. 0.95 ± 0.06) (a). Hippocampal BDNF mRNA levels were significantly lower in the UNO group than in the CONT group (0.55 ± 0.09 vs. 0.79 ± 0.13) (b). Box edges represent the upper and lower quantiles, with median values shown by the middle line in each box. Whiskers represent maxima and minima. *$p < 0.05$. Box edges represent the upper and lower quantiles with the median values shown by the middle line in each box. The whiskers represent the maxima and minima. Abbreviations: TrkB, tyrosine kinase receptor B; BDNF, brain-derived neurotrophic factor; CONT, control group; UNO, unilateral nasal obstruction group

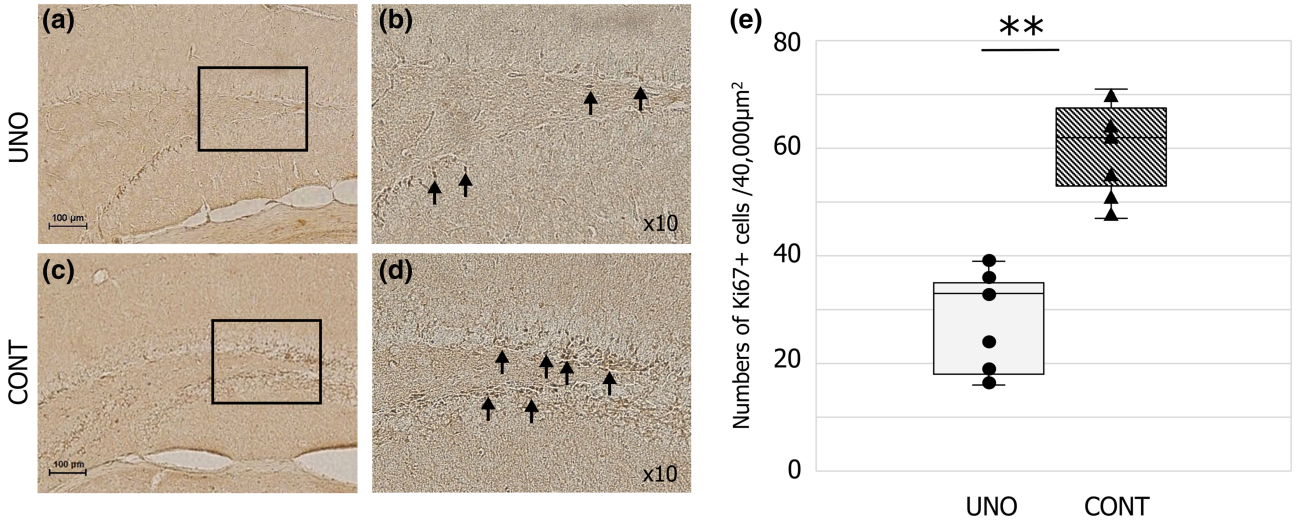

FIGURE 6 Neurogenic ability in the dentate gyrus. Representative images of Ki67 immunostaining in UNO (a, c) and CONT (b, d) groups. The number of Ki67-positive cells in the DG region of the hippocampus was significantly lower in the UNO group than in the CONT group (26.44 ± 7.08 vs. 60.55 ± 7.73) (e). *$p < 0.05$. scale bar (a, b): 100 μm. Box edges represent the upper and lower quantiles, with the median values shown by the middle line in each box. Whiskers represent the maxima and minima. Abbreviations: DG, dentate gyrus; CONT, control group; UNO, unilateral nasal obstruction group

Our findings suggest that UNO modulates the hippocampal Wnt/β-Catenin pathway, the upstream of BDNF production during the growth period. There was a significant positive correlation between SpO_2 levels and TrkB mRNA expression.

Many studies have examined the effects of nasal respiratory disorders on morphological and functional changes in the craniofacial region in humans (Góis et al., 2008). Nasal congestion may lead to craniofacial changes, including maxillary protrusion and adenoid

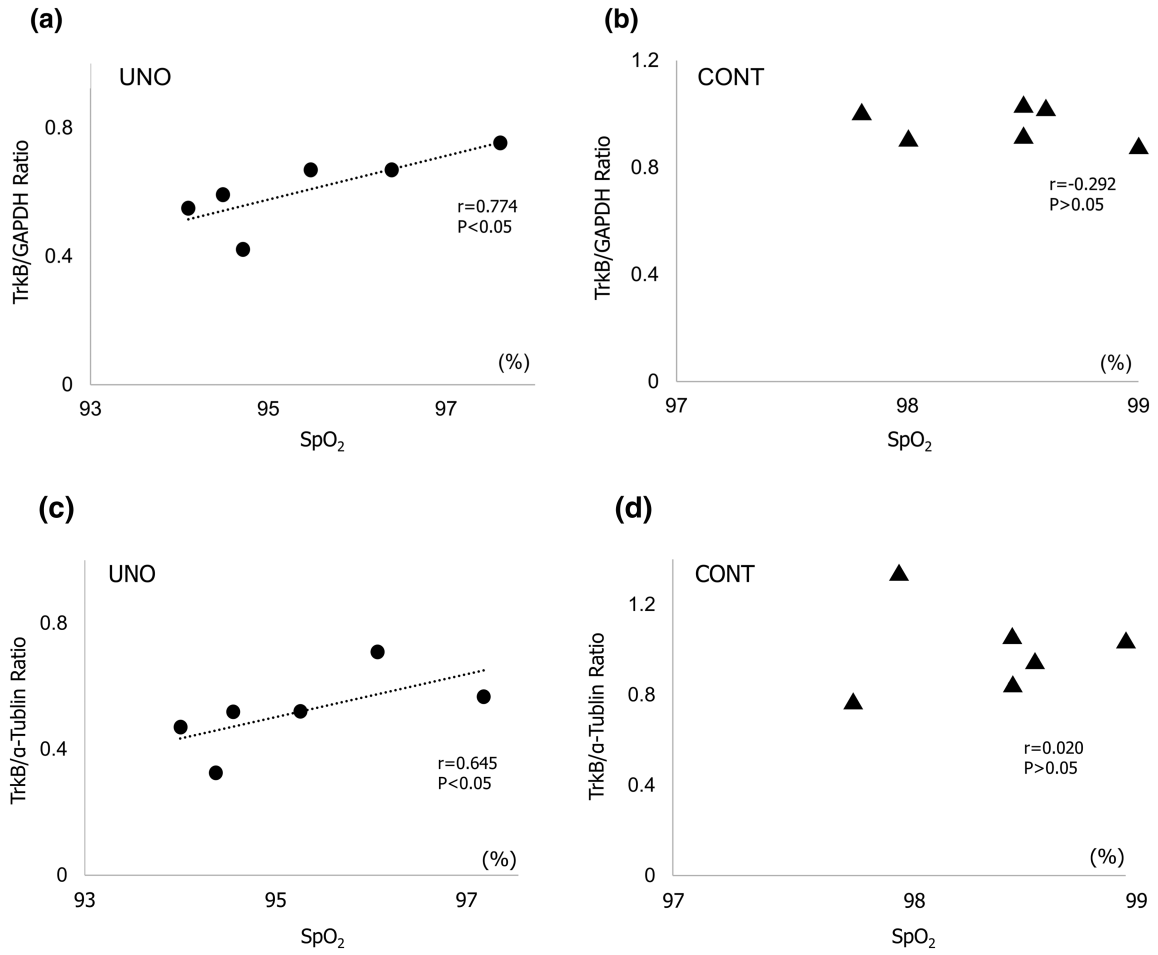

FIGURE 7 Correlations between SpO_2 levels and TrkB mRNA expression and protein levels of TrkB in the UNO and CONT groups. There was a significant positive correlation between SpO_2 levels and TrkB mRNA expression in the UNO group ($p = 0.017$, $r = 0.774$) (a). There was no significant correlation between SpO_2 levels and TrkB mRNA expression in the CONT group ($p = 0.475$, $r = -0.292$) (b). There was a significant positive correlation between SpO_2 levels and protein levels of TrkB in the UNO group ($p = 0.036$, $r = 0.645$) (c). There was no significant correlation between SpO_2 levels and protein levels of TrkB in the CONT group ($p = 0.960$, $r = 0.020$) (d). Abbreviations: TrkB, tyrosine kinase receptor B; SpO_2, blood oxygenation saturation; CONT, control group; UNO, unilateral nasal obstruction group

facies (Pirilä-Parkkinen et al., 2009) (Harari et al., 2010). Indeed, children with adenoid hypertrophy, allergic rhinitis, and oral respiration caused by nasal congestion exhibit deficits in their comprehensive, mathematical, and academic abilities and working memory compared with healthy children (Walker et al., 2007); (Fensterseifer et al., 2013) (Kuroishi et al., 2015). Thus, nasal obstruction not only causes morphological changes in the maxillofacial region but can also affect memory and learning functions. However, only a few studies have examined changes in memory and learning abilities related to nasal obstruction. Ogawa et al. (2018) found that the expression of phospho-p44/42MAPK and TrkB proteins involved in the BDNF/TrkB signals decreased in the hippocampus in the UNO group, while the expression of BDNF proteins increased.

Prior to this study, we proposed that UNO increases the expression of BDNF. However, the mechanisms underlying changes in protein expression of BDNF and TrkB in the UNO group have not been clarified, and little is known about the factors contributing to changes in BDNF/TrkB signalling. Determining the cause of the increase in BDNF and decrease in TrkB in the UNO group will help elucidate the effects of impaired nasal breathing on growth. We observed a decrease in SpO_2 levels, TrkB mRNA, and TrkB protein in the UNO group. This is consistent with a previous report that hypoxic conditions led to downregulation of hippocampal TrkB (Das et al., 2018). These findings suggest that chronic SpO_2 reduction due to UNO during the growth period may cause the downregulation of TrkB protein and mRNA in the hippocampus. Moreover, BDNF protein

expression in the mouse hippocampus was increased in the UNO group compared with the CONT group. There are two possible mechanisms underlying the increased expression levels of the BDNF protein associated with UNO. First, the increase in the extent of BDNF protein production may occur, and BDNF released excessively into the synaptic cleft could not bind to TrkB. Second, the decrease in TrkB expression might precede BDNF production, and consequently, BDNF could not bind to receptors, thus, overflowing into the synaptic cleft. Therefore, we focused on the Wnt/β-Catenin pathway to determine whether the increase in total BDNF protein levels was due to BDNF overproduction or whether excessive BDNF was present in the synaptic cleft.

The Wnt/β-Catenin pathway plays a wide range of important roles in various embryonic and adult neuronal functions, including neuroprotection, neuronal differentiation, and synaptogenesis. Recent studies have suggested that the Wnt/β-catenin pathway contributes to hippocampal learning and memory process and that hippocampal Wnt/β-catenin signalling is dysregulated in neurodegenerative diseases, such as Parkinson's disease, Huntington's disease, and Alzheimer's disease (Godin et al., 2010; Toledo & Inestrosa, 2010) (Berwick & Harvey, 2012). Consistently, obstructive sleep apnoea has a relatively high prevalence among patients with neurodegenerative diseases (Ancoli-Israel & Kripke, 1991; Kaminska et al., 2015) (Piano et al., 2015). In addition, BDNF levels increase in damaged glial cells and the Wnt/β-Catenin pathway is similarly activated (Garcia et al., 2018). It has been reported that Wnt signalling was enhanced after BDNF upregulation and suppressed after BDNF downregulation (Yang et al., 2015). Moreover, Wnt3a, a part of the Wnt family of proteins, induces BDNF expression in neurons (Yi et al., 2012) and chronic hypoxia reduces the expression of β-catenin in the mouse hippocampus (Pan et al., 2016). In this study, we investigated the protein expression of Wnt3a, Dkk-1, and β-catenin to examine whether unilateral nasal respiratory distress during growth modifies the Wnt/β-Catenin pathway. We observed that Wnt3a and β-catenin protein expression was significantly reduced in the UNO group compared with the CONT group. In summary, these findings suggest that UNO during the growth period modulates the Wnt/β-Catenin pathway in the mouse hippocampus and the mRNA expression level of BDNF was reduced. In addition, TrkB mRNA production was also reduced, and there was a significant positive correlation between SpO_2 levels and TrkB mRNA expression in the UNO group. Previous reports indicate that the expression of hippocampal TrKB is decreased in the IH model (Das et al., 2018). This suggests that the decrease in hippocampal TrkB expression may be due to the decrease in SpO_2 levels. In other words, UNO during growth decreases the expression of TrkB. Therefore, it is speculated that BDNF might overflow into the synaptic cleft because of the downregulated TrkB and increased BDNF total protein levels. The modulation of the Wnt/β-catenin pathway by UNO during growth resulted in a significant decrease in BDNF mRNA, which in turn decreased

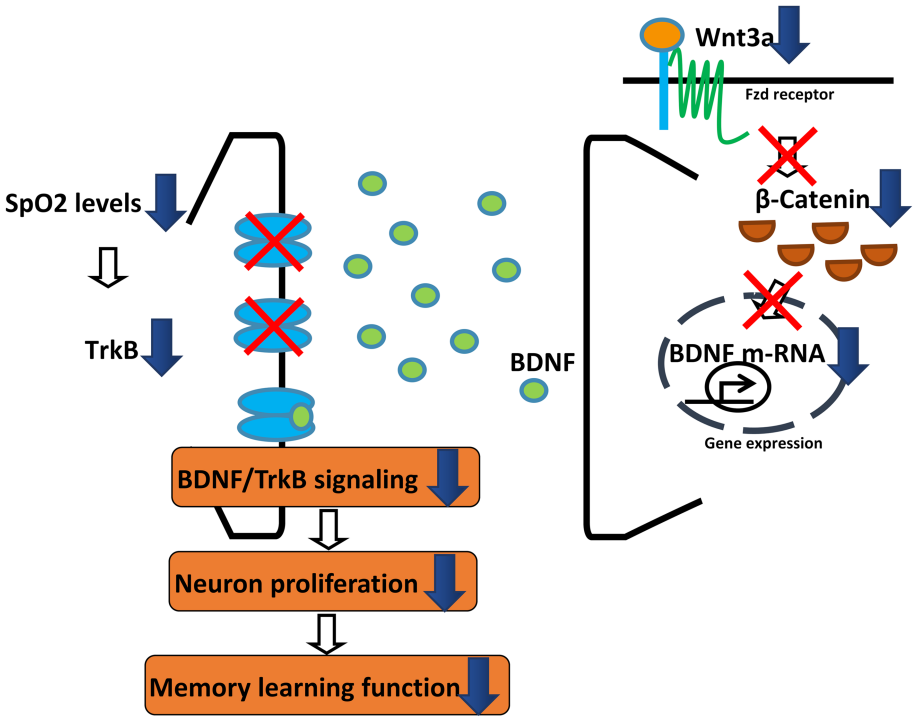

FIGURE 8 Schematic diagram of the molecular mechanism of this study. For interpretation, refer to the text

BDNF production. The decrease of hippocampal Wnt3a and β-Catenin, downstream of the Wnt/β-Catenin pathway is considered to regulate excessive BDNF.

The production of TrkB and BDNF was decreased in the hippocampus in the UNO group. Consequently, the expression of BDNF/TrkB signalling also decreased. Previous studies have demonstrated that UNO causes a decrease in the number of hippocampal neurons (Ogawa et al., 2018). However, few studies have focused on the relationship between the decline in memory learning function and the proliferative capacity of hippocampal neurons in the UNO model. Our current findings demonstrated a decrease in the number of Ki67-positive neurons in the dentate gyrus in the UNO group. It has been proposed that a decrease in the number of hippocampal Ki67-positive cells causes a decrease in memory learning function. Therefore, the decrease in the number of Ki67-positive cells caused by UNO during the growth period may induce memory-learning dysfunction.

It is suggested that the decrease in mouse memory learning function by UNO during the growth period is due to the decline in TrkB expression associated with a decrease in blood oxygenation levels. In addition, our study confirmed that the Wnt/β-catenin pathway is located upstream of BDNF production in the mouse hippocampus and is significantly involved in the proliferation and maintenance of hippocampal neurons (Figure 8).

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CONFLICT OF INTEREST

The author declares no conflicts of interest.

AUTHOR CONTRIBUTIONS

HI, HO, and SK conceived and designed the study. HI, TO, YA, and CK performed the behavioural and biochemical analyses. PTA and AF performed the histological analysis. HI and HO wrote the initial draft of the manuscript. TO revised and finalized the manuscript. All authors have read and approved the final version of the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ejn.15547.

DATA AVAILABILITY STATEMENT

The datasets used and analysed in this study are available from the corresponding author upon reasonable request.

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